

HD 2004: Changes, Advances, and Good News (CAG)_n

August 12-15, 2004.
Royal Sonesta Hotel
Cambridge, Mass.

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Abstract

One of the most striking features of Huntington's disease (HD) is its multiple faces, its varied effects on behavior, anatomy, cellular and molecular physiology. Covering an impressive amount of ground in three days, participants at the HD 2004: Changes, Advances, and Good News (CAG)_n meeting tackled this multi-faceted complexity. At the molecular level, they described new insights into the toxic structures that mutant huntingtin can adopt, the effects of post-translational modifications, and the potential importance of clearance pathways. Participants also examined new data on the normal functions of huntingtin which may contribute to HD pathology and to the selective vulnerability of cortical and striatal neurons. A striking array of cellular and molecular mechanisms were proposed to contribute to HD pathogenesis, including transcriptional alterations, cellular transport disruptions, mitochondrial dysfunction, and altered calcium signaling. In addition, the importance of subcellular, cellular and tissue context was discussed.

A wide range of efforts towards developing therapeutic agents was also presented. Participants described candidate therapies that target the primary sources of HD pathology, including the use of intrabodies and RNA interference, as well as compounds that ameliorate HD-associated symptoms. Of particular interest, clinical trials using tetrabenazine indicate this well-studied compound is particularly well-suited to control the debilitating chorea associated with HD. Participants also discussed approaches to accelerate the flow of candidate therapies through the drug discovery pipeline, and analyzed ways to streamline clinical trials and increase their power. The identification of novel HD biomarkers was highlighted as particularly important.

Introduction

Over 300 scientists, giving 196 presentations, attended the meeting "HD 2004: Changes, Advances, and Good News (CAG)_n"—100 more than in the previous meeting two years ago.

Eleven years ago, when the mutation underlying HD was first identified, no one could have predicted the difficulty of the road ahead. The single, well-defined dominant mutation—a CAG expansion in the gene encoding "Interesting Transcript 15" (IT15), now known as huntingtin—belied a web of biological complexities. No one could have anticipated mutant huntingtin's stubborn resistance to reveal its conformations, nor its strikingly promiscuous amino terminus, capable of interacting with over 150 protein partners. No one could have foreseen mutant huntingtin's remarkable ability to spawn a variety of aggregate forms and perturb dozens of cellular pathways. Nor did anyone expect that a protein expressed throughout the body, would cause conspicuous damage to only a subset of neurons in the brain.

As Nancy Wexler stated in her opening remarks, HD is not an easy research subject to tackle. Researchers who have attempted to uncover its secrets have often been overwhelmed by its intricate biology. Yet they have persevered and, indeed, inspired others to join them. In a heartfelt address to the HD community, Wexler thanked participants for their dedicated efforts and their relentless pursuit of an understanding of, and eventually a cure for, HD.

Mutant huntingtin

It has long been known that an expanded poly-glutamine tract in the huntingtin protein lies at the core of HD pathology. How this expansion causes toxicity, however, remains elusive. Participants discussed recent progress in identifying the toxic configurations of mutant huntingtin, understanding how they are generated, and elucidating how they mediate toxicity.

Polyglutamine structures and aggregate formation

Michelle Poirier presented data suggesting that mutant poly-glutamine adopts a beta-structure upon conversion to its toxic form. Previous *in vitro* studies by Wetzel and colleagues indicated that expanded poly-glutamine can adopt a beta-strand/beta-turn structure. To test this model in living cells, Poirier transfected a neuronal cell line and primary cortical neurons with constructs encoding huntingtin exon 1 containing proline substitutions. She observed that when the prolines were within the putative beta-strands, and thus disrupted their conformation, huntingtin did not aggregate as assessed biochemically and by immunofluorescence. In addition, TUNEL staining and morphological examination of cells expressing these constructs revealed a marked decrease of toxicity. Thus, the results support the proposal that the beta-strand/beta-turn structure is required for aggregation and toxicity.

On the other hand, Valerie Daggett proposed an alpha chain configuration for expanded poly-glutamine which might cause toxicity through the formation of an alpha-pleated sheet secondary structure. Daggett noted that her group has performed molecular dynamics simulations *in silico* that predict the formation of an alpha-extended chain in various amyloidogenic proteins. The transition to this alpha chain exhibits length and temperature dependence consistent with experimental observations. In addition, the model helps explain the mechanism of action of a newly identified inhibitor of fibril formation. Daggett enjoined experimentalists to collaborate with her to further test the model and extend her current efforts to devise and carry out alpha-sheet detection assays.

Tomas Ding presented observational data on huntingtin structure. Using atomic force microscopy, he has identified various fibrillar and pre-fibrillar structures formed by mutant huntingtin exon 1 *in vitro*. Within 2 hours of incubation at room temperature, Ding observes spherical structures similar to those detected with other aggregating proteins such as alpha-synuclein. He also observes novel annular assemblies. Over time, these species disappear giving way to fibrils of increased height. To assess the toxicity of the pre-fibrillar structures, Ding is devising methods to obtain larger quantities of the structures and rapidly introduce them into cells.

Proteolysis of huntingtin and aggregate formation

As noted by Steven Finkbeiner and Christopher Ross, a key challenge will be determining the relative contributions of these proposed and observed configurations to the disease process. Ross added that it will also be important to establish whether covalent modifications, in particular cleavage, precede conformational changes.

Caspase cleavage of huntingtin is one of the best studied of these modifications. It has been well characterized *in vitro* and caspase-derived fragments of huntingtin have been identified in human brains early in the progression of HD. To test the relative importance of huntingtin cleavage by caspases-3 and -6, Rona Graham described mutating these cleavage sites in the YAC model of HD. The mutations didn't otherwise interfere with huntingtin function. To make appropriate comparisons between HD lines and HD lines with caspase site mutations, mice with similar expression levels were paired. The data indicated that mutations in the caspase-6, but not the caspase-3, site preserved normal striatal volume. As noted by Michael Hayden, the difference in length between the amino-terminal fragments generated by caspase-3 and -6 is only 34 amino acids. The findings are consistent with other studies indicating that caspase-6 is elevated in human striata and caspase-6 is activated early in HD mice.

On the other hand, Yvon Trottier and Ross's group have identified fragments possibly generated by aspartyl proteases, that are associated with toxicity. The fragment described by Trottier is known as Cp-A and is only 129 amino acids long. Trottier identified Cp-A, and its relative Cp-B, in nuclear inclusions of the NG108 neuroblastoma cell line and has confirmed its presence in a rat model of HD. The fragments appear to be key to the formation of nuclear inclusions. In addition, Trottier reported that the lysosomal enzyme cathepsin D co-localizes with huntingtin in the cell and, *in vitro*, can accelerate the generation of Cp-A and Cp-B. The acceleration, however, is only observed at neutral pH, suggesting that limited and local delivery of cathepsin D into the cytoplasm could result in huntingtin cleavage and the release of fragments that tend to aggregate. Other endo-peptidases may also be involved, since cathepsin D-deficient cells generate Cp-A, albeit in lesser amounts.

Understanding the roles of particular huntingtin fragments in mediating toxicity and aggregation may be more complicated than previously expected, however. Examining a serendipitously generated mouse that expresses a 117 amino acid fragment of huntingtin including exons 1 and 2, named "shortstop", Hayden made a surprising discovery that highlights the need for a deeper understanding of huntingtin fragments and their toxicity. Previous findings indicated that exon 1 and fragments similar to shortstop, such as Cp-A, induce inclusion formation and severe neuropathological and motor phenotypes. Consequently, Hayden was not surprised when he observed accelerated nuclear inclusion formation,

increased density of aggregation foci, and a loss of regional specificity in shortstop mice compared to equivalent full-length models of HD. However, in contrast to both full-length and other truncated models of HD, shortstop mice showed no rotarod deficits at 12 months of age, no changes in brain weight, and no decreases in striatal volume and neuron numbers. In addition, they were less, rather than more, susceptible to quinolinic acid excitotoxicity. The mice's overall phenotype and behavior appears to be normal, at least until the age of 12 months.

Hayden notes that there are still many unanswered questions surrounding this paradoxical result. However, he thinks the data do not exclude the importance of toxic fragments in HD, but rather suggest that exact fragment sequences are of critical importance in mediating toxicity. Indeed, a difference of just two amino acids in the A β peptide of Alzheimer's disease can mark the difference between toxicity and non-toxicity. Kimberly Kegel added that generation of a toxic huntingtin fragment may depend on huntingtin's cleavage by membrane-associated proteases. A lack of membrane association sequences in shortstop may preclude the generation of the precise sequence required for toxicity.

Hayden's results also suggest that neuronal inclusions are not always pathogenic. Several participants considered it important to examine shortstop's aggregates in more detail. Ethan Signer and Paul Patterson, for example, suggested using conformation-specific antibodies to probe shortstop's aggregates and compare them to those generated in full-length models. Hayden added that the path to aggregation and toxicity is probably not a single, linear process and, therefore, the physiological interpretation of his findings will be complex. One possibility is that the shortstop fragment lacks critical sequences that normally help determine huntingtin's amino-terminus structure and its associated toxicity. Carl Johnson, for example, proposed that shortstop fragments may self-associate so quickly that they spend virtually no time in the oligomeric form that has been associated with toxicity.

Hayden urged participants to define the exact sizes of the fragments they study, use appropriate controls, and bear in mind that full-length models of HD are not equivalent to fragment models. In addition, Fred Goldberg cautioned that it was easy to attribute the generation of huntingtin fragments to specific proteases, without considering that exonucleases can produce a wide variety of fragments. In addition, the proteasome has several proteolytic activities that can be confused with those of other proteases, including calpain-like and caspase-like activities.

Aggregate formation and the importance of molecular and cellular context

Another post-translational modification that appears to be important for determining mutant huntingtin's toxicity is SUMOylation, a post-translational modification in which SUMO-1 (small ubiquitin-like modifier) is covalently attached to lysine residues. Joan Steffan presented data indicating that, in cell culture, mutant huntingtin can be modified by SUMO-1 or ubiquitin in a mutually exclusive manner. Genetic reduction of SUMOylation in an HD *Drosophila* model suppresses lethality and neurodegeneration. Furthermore, elimination of mutant huntingtin's lysine residues that are targeted by SUMO-1 significantly ameliorates HD pathology. Several mechanisms may contribute to these effects. SUMOylation enhances mutant huntingtin's stability and alters its aggregation properties. In addition, SUMOylation may mask a cytoplasmic-targeting domain present in huntingtin's amino-terminus and thus allow nuclear localization of mutant huntingtin. Steffan noted that the first 17 amino acids of huntingtin, which contain lysines that can be SUMOylated, can target proteins to the cytosol even in the presence of a strong nuclear localization sequence (NLS).

Another study that suggests mutant huntingtin's ability to get into the nucleus and form inclusions may be key to its toxicity was presented by Dan Goldowitz. His group discovered a colony of R6/2 mice that has hyper-expanded CAG repeats (over 250) but, paradoxically, these mice live much longer than regular R6/2 mice. The long-lived mice, R6/2x, share behavioral and neuropathological deficits with R6/2 mice, but their cortical neurons have predominantly cytoplasmic aggregates with very few nuclear inclusions. When Goldowitz crossed R6/2 and R6/2x mice, the offspring had both nuclear and cytoplasmic inclusions, and died slightly earlier than R6/2 mice. Although other studies, including Hayden's shortstop results, suggest that nuclear localization is not the *sine qua non* of toxicity, it is possible that the hyper-expanded repeats may contribute to the extension of the animals' survival by preventing huntingtin fragments from entering the nucleus. Goldowitz also noted that nuclear and cytoplasmic aggregates probably act synergistically to cause pathology. As pointed out by Kurt (Kenneth) Fischbeck, understanding the how, where, and when of aggregation may be key to elucidating its link to toxicity.

Wetzel presented data describing how cellular and protein context affect the aggregation process. His team showed that a stretch of 10 prolines placed next to polyQ reduced aggregation. In addition, he identified several cellular factors that affect aggregation. As previously reported for alpha-synuclein, Wetzel found that aggregation of poly-glutamine proteins increases when other macromolecules are present at high concentrations and there is little space for the diffusion of large molecules, a situation that occurs in cells and is known as molecular crowding. Moreover, he reported that the molecular

chaperone Hdj extends the lag time of poly-glutamine aggregation at low stoichiometric doses, possibly acting through the disruption of early nucleation sites. Wetzel also presented data indicating that short poly-glutamine peptides amplify the nucleation kinetics of longer polyglutamine chains. This finding may help explain some observations from animal models and human genetics.

A window into where and when poly-glutamine recruitment into aggregates occurs *in vivo* was presented by Alex Osmand. Osmand used biotinylated poly-glutamine peptides at physiological concentrations to detect aggregation foci--sites that can actively recruit monomeric poly-glutamine molecules. Based on observations in human cortical tissue, he suggested foci first appear in the cytoplasm, grow in size, and then are transported to the axonal pole, ultimately giving rise to neuropil aggregates which do not recruit additional poly-glutamine molecules. Applying the technique to animal models of HD--YAC128 mice, Q140 knock-in mice, and a rat model expressing a fragment of mutant huntingtin--however, revealed a great degree of heterogeneity between human HD and the models, as well as between models. Several brain areas were affected differentially. In addition, in rats and mice, but not in humans, small aggregates which retain the ability to recruit poly-glutamine appeared to be transported rapidly into projection areas. Particularly large numbers of neuropil foci were observed in rat.

Using similar methods, Stephan von Hörsten examined the temporal relationships between the appearance of foci, aggregates, and behavioral symptoms. Interestingly, several behavioral alterations significantly preceded the appearance of aggregation foci and aggregates in transgenic rats carrying a mutant huntingtin fragment including amino acids 1-654 (corresponding to 1-684 in the human sequence). In particular, a reduction in ultrasonic vocalizations was evident as early as 15 days after birth, and a reduction in anxiety occurred at 4 weeks of age, shortly after increased motor activity. Aggregation foci, however, were detectable at 6 months of age in the cortex, and at 9 months in the thalamus and ventral striatum. Aggregates appeared even later, as revealed by the 1C2 antibody, followed by cell loss.

Antibodies emerged as a powerful tool to monitor different huntingtin aggregates. Patterson and his colleagues, for example, have generated recombinant antibodies against several epitopes of mutant huntingtin exon 1. Strikingly, various antibodies against the poly-glutamine region stained tissues and subcellular compartments differentially, indicating that the conformation of poly-glutamine varies according to its location. Patterson also observed that antibodies generated against aggregated huntingtin stained the nucleus, whereas most other antibodies preferentially stained the cytoplasm. Working with Wetzel, Patterson has recently generated antibodies against flexible protofibrils with a range of antigenic preferences--some are generic for amyloid structures, while others bind more specifically to poly-glutamine stretches or to particular amyloid proteins. In addition, some antibodies prefer aggregated protein forms while others prefer soluble forms. Curiously, these *in vitro* preferences are not always reflected in *in vivo* staining patterns.

Charles Glabe has also developed antibodies that promise to advance the understanding of huntingtin aggregation and toxicity. His group generated conformation-specific antibodies that recognize soluble oligomers of various amyloidogenic proteins, including poly-glutamine proteins. The antibodies inhibit toxicity *in vitro* and suggest that a wide variety of amyloids adopt common soluble, oligomeric configurations that may mediate similar mechanisms of toxicity. Glabe said he was struck by this commonality and predicted that understanding the pathological mechanisms of one disease would illuminate those of others. Glabe is now working with Leslie Thompson and Larry Marsh to assess the presence of huntingtin oligomers *in vivo*. Early findings indicate that the antibody indeed detects huntingtin exon 1 in *Drosophila* tissues.

Clearance

Complementing the studies of huntingtin aggregation, Ai Yamamoto and Prasanna Venkatraman presented findings on huntingtin clearance. Previous studies by Yamamoto using a mouse model of HD in which the synthesis of mutant huntingtin can be turned off, revealed that the formation of inclusions is a reversible phenomenon. However, the process of clearance is very slow, which may be responsible for the gradual accumulation of aggregates, resulting in a slow onset of the disease. To examine the pathways underlying clearance, Yamamoto used microarray analysis to identify genes that are upregulated in cell lines expressing expanded polyglutamine proteins. She then used siRNAs to knock down 56 of the upregulated genes and assess their effects on aggregate clearance. The approach identified lysosomal genes, vesicle transport genes and autophagy genes as required for clearance. The latter are of particular interest because autophagy can be potentiated to enhance clearance, without increasing cell death. In addition, Yamamoto noted that autophagy can be induced by activation of IRS2, a target for type 2 diabetes. This may benefit HD therapy development because of the large amounts of money and effort that are being invested in type 2 diabetes which will probably require long-term treatments like HD.

Another key aspect of huntingtin clearance, the digestion of huntingtin molecules by the proteasome, was discussed by Venkatraman. She and her colleagues discovered that, although eukaryotic proteasomes can cleave single glutamine-glutamine bonds, they are unable to cut poly-glutamine stretches. Thus, when degrading poly-glutamine proteins, eukaryotic proteasomes release undigested poly-glutamine peptides. Normally, these fragments are probably hydrolyzed by unidentified cellular peptidases. But because the poly-glutamine repeats of poly-glutamine diseases exceed the lengths of normal proteasome products (2-25 residues), they may linger for longer and more readily induce aggregation. In addition, occasional failures of these long fragments to exit the proteasome may interfere with proteasome function. Identifying the cellular peptidases that degrade polyglutamine fragments could provide a handle for ameliorating mutant huntingtin toxicity.

Normal functions of huntingtin

Because HD is an inherited autosomal dominant disease, expressing similarly in heterozygotes and homozygotes, the huntingtin mutation has commonly been expected to act as a gain-of-function mutation. Indeed, as noted by Marcy McDonald, a single copy of mutant huntingtin appears to be capable of rescuing wildtype huntingtin function. New findings reported by Songshan Jiang are also consistent with a gain-of-function effect. Jiang applied siRNA to knock down wildtype huntingtin in striatal cells, and observed alterations in cell function, but they were different to those caused by mutant huntingtin expression. BDNF and ATP levels which are usually disrupted in HD, for example, were similar to controls. It is not surprising then that, in the context of HD, studying huntingtin's normal function has received less attention than studying the toxicity of the mutant protein.

Increasing evidence indicates that loss-of-function effects, however, may also be important in HD pathology. Mutant huntingtin may mediate some of its toxic effects through the disruption of wildtype function. For example, as noted by Scott Zeitlin, mutant huntingtin may sequester the wildtype protein, induce its proteolysis, or form dysfunctional complexes with other proteins that normally interact with wildtype huntingtin. In addition, as pointed out by Elena Cattaneo and Juliana Woda, the regional vulnerability that characterizes HD might be explained by huntingtin's normal functions. And as noted by Anton Reiner, understanding huntingtin's normal function may aid developers of new therapies to avoid disrupting critical huntingtin functions.

A growing list

A multiplicity of functions for wildtype huntingtin were proposed. MacDonald noted that huntingtin is comprised almost entirely of HEAT repeats, a 40 amino acid motif. Stretches of HEAT repeats (called HEAT domains) are often involved in mediating protein-protein interactions such that huntingtin is likely to act as a scaffolding protein. Indeed, Bob Hughes who is conducting comprehensive searches for huntingtin interacting proteins reported his group has identified approximately 170 interacting proteins using systems-scale yeast-two-hybrid assays. Many interactors are either nuclear proteins, mitochondrial proteins, or proteins involved in vesicular traffic. In addition, he found six novel interactors with a preference for mutant huntingtin, including the glycolytic enzyme pyruvate kinase. There is a high degree of connectivity between interactors. In addition, mass spectroscopy analysis of pull-down complexes indicates that huntingtin is likely to function as part of multi-protein assemblies. Of particular interest, huntingtin may be part of the sarcoplasmic reticulum junctional complex in muscle—an intriguing finding given that the mutation underlying a disorder which is clinically very similar to HD, Huntington disease-like 2, maps to a gene encoding a member of this complex, junctophilin-3. Hughes also observed that huntingtin is associated with the SNARE vesicle complex.

Supporting huntingtin's role in vesicular transport, Frederic Saudou reported that huntingtin specifically enhances the transport of vesicles containing brain-derived neurotrophic factor (BDNF) along microtubules. Saudou examined the dynamics of BDNF-eGFP-containing vesicles in real time by 3D videomicroscopy. He found that wildtype huntingtin increased the velocity of the vesicles and decreased their pausing time. The effect required huntingtin associated protein-1 (HAP1) and the p150^{Glued} subunit of dynactin, an essential component of microtubule motors. Expressing mutant huntingtin or reducing wildtype huntingtin using siRNA dampened BDNF transport. In addition, transport deficits induced by mutant huntingtin resulted in the loss of neurotrophic support and neuronal toxicity. These effects are specific: mitochondrial movement, which also occurs via microtubules, is not similarly affected. Saudou suggested that HAP-1 might mediate cargo specificity. He is now examining the extent of this specificity, evaluating the transport of other cargoes in addition to BDNF.

Insights into how huntingtin may mediate membrane-associated functions were provided by Kegel, who made huntingtin constructs with site-directed mutations to identify huntingtin's membrane-binding regions. Using biochemical assays and immunofluorescence, Kegel found that a region in the N-terminus rich in cationic amino acids, and predicted to

be high in alpha-helical structure by computer modeling (spanning amino acids 171 to 287) is important for huntingtin membrane association. Furthermore, a hydrophobic helix in this domain can insert into synthetic lipid bilayers as assessed by differential scanning calorimetry, suggesting it might act as a membrane anchor. The charged regions on either side of the helix, however, are also important for membrane association. A protein-lipid overlay assay revealed that N-terminal huntingtin fragments in lysates bind to acidic phospholipids.

Participants also discussed huntingtin's potential functions in the nucleus. Ray Truant noted that his group has observed huntingtin moving in and out of the nucleus which may be related to its previously proposed association with the NF-kappa B signaling pathway. Patterson added that examining this association may be particularly informative, since mutant huntingtin's association with NF-kappa B is altered.

Another role for huntingtin in the nucleus was presented by Cattaneo. Previous studies from her group indicate that wildtype huntingtin sequesters the repressor element-1 transcription factor (REST), thus inhibiting the activity of the Neuronal Restrictive Silencer Element (NRSE), a silencer located in the promoters of many neuron-specific genes, including the BDNF gene. To examine this inhibition in greater detail and ascertain the effects of mutant huntingtin, Cattaneo has now used chromosome immunoprecipitation (ChIP) assays to monitor REST binding to BDNF and synapsin 1 NRSEs. In both cultured cells and *in vivo*, REST binding is increased in neurons expressing mutant huntingtin as compared to controls. In addition, the occupancy of REST increases over time in cortical neurons of R6/2 and *Hdh* mice as the disease progresses. To systematically assess the effects of mutant huntingtin on all NRSE-containing genes, Cattaneo is now setting up genome-wide NSRE screens, in effect, conducting ChIP on chips.

Additional clues about the functions of wildtype huntingtin were presented by Edoardo Marcora and Jiang. Marcora reported that huntingtin and HAP-1 co-purify with the post-synaptic density protein PSD95 in PSD preparations, and decreased amounts of huntingtin are observed in PSD preparations from mutant mice. Huntingtin's function at this location, however, remains unknown. Jiang noted that knocking down huntingtin expression results in a corresponding decrease of certain huntingtin interactors, in particular symplekin, a protein involved in poly-adenylation and cell-cell junctions.

Probing huntingtin's role in development, Woda examined embryonic patterning and morphogenesis in huntingtin knockout mouse embryos. She reported that huntingtin, which is expressed ubiquitously during development, is required for proper posterior restriction of growth factor expression (FGF-8, nodal, Gsc) and node formation in early embryogenesis. The failure in restriction does not reflect a loss in anterior/posterior polarity, however, since deficient embryos show normal expression of markers of the anterior visceral endoderm, and express extra-embryonic signals, including BMP-4.

Open questions and future directions

In sum, it is clear that wildtype huntingtin can carry out multiple functions. This is not surprising, particularly in light of its abundance of HEAT repeats, which appear to always be involved in mediating protein-protein interactions. Indeed, as noted by Hughes, other HEAT-containing proteins, such as beta-catenin, have multiple, sometimes disparate functions. Nevertheless, many mysteries still surround wildtype huntingtin. For example, as noted by Patterson, it is unclear why huntingtin is so large—other scaffolding proteins are much smaller. Also, it is puzzling that practically all huntingtin partners identified to date bind to huntingtin's amino terminus. Hughes said his group spent two years searching for carboxy terminus partners, but was unable to find any. Michael Sauder noted that regions of huntingtin outside of exon 1 have a tendency to self-associate, based on expression of hundreds of constructs from several different species, and Patterson added that the carboxy-terminus may provide structure for the amino-terminus..

Participants also reflected on what should be required to classify a particular process as a "normal" huntingtin function. As noted by McDonald and Kegel, a complicating factor is context—huntingtin may have different functions depending on tissue, cell, and subcellular location. And as pointed out by Cattaneo, the type of evidence that each researcher considers appropriate to define a function depends on the researcher's expertise and methodology. As a cell biologist, for example, she defines function in terms of its contribution to cell survival. A chemist, on the other hand, may rely on *in vitro* assays. Jim Gusella used a car analogy to help clarify the definition of function: the function of a car is to get people from A to B, whereas its activities include going over bumps, speeding up and slowing down, and playing the radio—events that are easily measured.

An often useful indicator of biological function is evolutionary conservation. Tobin wondered whether examining the conservation of huntingtin domains may shed light on which activities are most relevant to huntingtin's functions. As noted by Johnson, one complication with this approach is that huntingtin's distribution across the phylogenetic tree is unexpected: slime molds have a huntingtin orthologue, yet *Caenorhabditis elegans*, which has a well-developed nervous system, does not.

In addition to discussing huntingtin's normal functions, participants discussed the potential effects of mutant huntingtin on wildtype huntingtin. Marcora wondered if mutant huntingtin can induce conformational changes in wildtype huntingtin, whether wildtype huntingtin localizes to aggregates, and how much mutant huntingtin is required to induce pathology. Jang-Ho Cha asked whether wildtype huntingtin is normally cleaved and, if not, could its cleavage in HD release fragments that act as dominant-negative effectors. A powerful tool that promises to enable the examination of these questions was presented by Zeitlin. His team is generating mice carrying epitope-tagged huntingtin alleles that allow the discrimination between normal and mutant huntingtin in tissue and cellular extracts. The tags do not seem to affect normal huntingtin function, nor expression. In addition, control experiments revealed no direct or indirect interaction of normal huntingtin with itself. Zeitlin is now setting up to characterize changes in the amount and cellular location of normal huntingtin during HD progression in mice carrying normal and mutant tagged alleles.

Participants had several additional suggestions for future directions. Cattaneo proposed using systems expressing different doses of wildtype and/or mutant huntingtin to evaluate whether loss of huntingtin function is important for the disease process. Fishbeck and Reiner proposed examining deficiencies in proteins or processes known to be associated with HD. For example, Fishbeck noted that mutations in dynactin result in neurodegeneration. Although the degeneration is not striatal-specific, it may shed light on what happens when mutant huntingtin disrupts cellular transport. Reiner proposed examining in striatal cells the effects of knocking out BDNF in the cortex. Another suggestion was to analyze huntingtin's post-translational modifications, in particular phosphorylation. Hughes emphasized the power of model systems such as zebra fish and *Drosophila*. Finally, Patterson urged participants to replace statements that describe huntingtin as a protein of unknown function in their articles, with descriptions of huntingtin as a multi-functional protein.

Mechanisms of pathogenesis in models of HD

A striking array of cellular and molecular mechanisms were proposed to contribute to HD pathogenesis. Participants presented data implicating transcriptional alterations, cellular transport disruptions, mitochondrial dysfunction, and altered calcium signaling. In addition, the importance of context was discussed. Cellular and molecular factors that can potentiate or ameliorate toxicity were noted for their key contributions to mutant huntingtin's effects. Another important point of discussion was whether the damage seen in specific brain regions is mediated by mutant huntingtin's direct effects on the regions' cells, or alternatively, by the protein's effects on other cells that interact with the region. Participants also discussed requirements and effective approaches for linking pathological mechanisms to HD, as well as their views for the future.

Transcriptional dysregulation

Several studies have shown that transcriptional alterations are associated with HD. Global assessments of transcription using microarrays, however, had been limited to animal models. Extending these studies to humans, Lesley Jones presented data from the HD Human Array Group on gene expression patterns in the cerebellum, cortex and caudate of post-mortem brains with relatively low grade HD pathology. The caudate showed the highest number and greatest magnitude of expression changes. Functional categories of genes that were significantly disrupted included neurotransmission and intracellular signaling. In contrast, genes involved in apoptosis, oxidative stress, and protein folding were not substantially affected.

Of particular interest, previous results from R6/2 mice overlapped significantly with the human caudate results, in contrast to full-length models. As noted by Jim Olson, this suggests human disease may be primarily driven by the toxic effects of huntingtin fragments. This finding generated considerable interest because it runs counter to the commonly held assumption that full-length models mirror human disease more closely than fragment models.

In addition, this study revealed that, although regional specificity has not been apparent in R6/2 mice, important differences between brain regions are evident in humans. In particular, Brodman areas 4 and 9 of the cortex were differentially affected. Area 4, which corresponds to the primary motor cortex and projects to the striatum, was the most affected cortical area and shared the most similarities with the caudate. As noted by Olson, such regional variations promise to shed light on the underpinnings of selective vulnerability.

Participants also discussed potential molecular mechanisms underlying HD's transcriptional dysregulation. Weiguo Zhai, for example, reported that the transcription factor Sp1 and two components of the basal transcription machinery, TAF_{II}5 and TFIIIF, are targeted by mutant huntingtin. The amino-terminus of mutant huntingtin appears to interact with TAF_{II}5 and TFIIIF, and the potency of its inhibitory effects on transcription is dependent on poly-glutamine length. Mutant huntingtin can interact with TAF_{II}5 and inhibit phosphorylation of one of TFIIIF's subunits, RAP74, *in vivo*. Zhai also noted that mutant

huntingtin and RAP74 compete for binding to TFIIIF's other subunit, RAP30. Indeed, GST pull-down experiments indicate that mutant huntingtin breaks up RAP74/RAP30 complexes. Consistent with these observations, RAP30 over-expression can protect primary striatal neurons from mutant huntingtin toxicity.

An in-depth examination of Sp1's role in HD was presented by Steve Hersch. Hersch observed increased levels of Sp1 in neuronal-like PC12 cells expressing mutant huntingtin, primary striatal cells from R6/2 mice, and brain tissue of R6/2 mice in the early stages of disease. He also observed Sp1 elevation when 3-nitropropionate (3-NP), a compound that mimics some of HD's pathology, was used to induce cell death in PC12 cells. To assess the effects of knocking down Sp1 on HD pathology, Hersch used siRNA, a heterozygous Sp1 knockout mouse, and mithramycin A, a DNA intercalating agent that inhibits Sp1 function. The three approaches yielded consistent results, showing that reduced levels of Sp1 ameliorate toxicity caused by either mutant huntingtin or 3-NP. In addition, when HD mice were crossed with Sp1 knockout mice, the resulting offspring did not experience the loss of dopamine D2 receptor mRNA which characterizes HD mice, and survived longer than their HD progenitors.

Using a *C. elegans* model of HD pathology, Alex Parker from the group of Christian Neri reported the identification of *sir-2.1*, a histone deacetylase (HDAC) which also deacetylates other cellular proteins, as a genetic modifier of poly-glutamine toxicity. They observed that *SIR2.1* overexpression reduces dystrophic axons and neuronal cell dysfunction. Conversely, *SIR2.1* inactivation potentiates cytotoxicity. On this basis, Neri's group is now evaluating the effects of manipulating sirtuins pharmacologically in *C. elegans* and mammalian systems.

A transcriptional regulator whose association with HD has been the subject of several studies, the CREB binding protein (CBP), was also discussed at the meeting. CBP is a transcriptional cofactor and histone acetyltransferase essential for neuronal survival. Previous reports indicate that mutant huntingtin may interfere with CBP function by sequestering CBP in nuclear inclusions, by inhibiting its HAT activity, and/or by promoting its degradation. Based on these findings, Juan Botas examined whether increasing CBP levels could ameliorate HD pathology. Botas used *Drosophila* lines expressing either the polyglutamine protein ataxin-1 or mutant huntingtin and various CBP constructs. Surprisingly, he observed that increased levels of CBP enhance neurodegeneration and motor deficits. CBP deletion mutants revealed that the negative effects were mediated by CBP's polyglutamine region. Botas concluded that increasing CBP levels is probably not a good therapeutic option. However, Fishbeck cautioned that his group has done similar experiments and obtained opposite results. He noted that transcriptional regulation is complicated, and that differences in the stoichiometry of regulators in different systems could account for disparate results. Adding further to the complication of CBP's role in HD, a recent report indicates that CBP-mediated transcription is enhanced, instead of diminished, in the mouse R6/2 model of HD. Olson proposed that preventing interactions between CBP and mutant huntingtin may be a better therapeutic approach than altering CBP levels.

Yet another transcriptional alteration was presented by Marta Valenza. She and her colleagues found that the expression of several genes involved in cholesterol synthesis is decreased in cell and animal models of HD, as well as in the human HD brain. Cholesterol is of particular interest because it is produced locally in the brain and is required for synaptic remodeling. Interestingly, all of the affected genes are regulated by the SREBP transcription factor, and an SRE-Lac reporter construct confirmed that cells expressing mutant huntingtin are impaired in SREBP-mediated transcription. Moreover, localization studies revealed that SREBP's translocation to the nucleus is impaired in HD cells, and cholesterol administration rescued cultured striatal cells from death. Olson suggested studying the effects of experimentally reducing cholesterol synthesis on the spine morphology of medium spiny cells. In addition, Anne Messer proposed looking into HD patient records to assess the effects of cholesterol-lowering drugs, such as statins.

Alterations in cellular transport

One factor that may contribute to mutant huntingtin's effects on transcription is the abnormal accumulation of its fragments in the nucleus. Xiao-Jiang Li presented data suggesting how this may occur. Based on yeast-two hybrid studies indicating that amino-terminal huntingtin fragments interact with Tpr, a nuclear pore protein, Li asked whether expanded polyglutamines could affect this interaction and lead to a disruption in nuclear transport. *In vitro* binding studies and immunoprecipitations of endogenous Tpr revealed that, indeed, mutant huntingtin interacts less strongly with Tpr than wildtype huntingtin. Li also observed that knocking down Tpr with siRNA increased huntingtin accumulation in the nucleus. In addition, huntingtin deletion mutants lacking the Tpr binding site accumulated abnormally in the nucleus. Li's current model is that both wildtype and mutant huntingtin are cleaved and some of the resulting fragments enter the nucleus, but whereas the wildtype fragments are rapidly exported, the mutant fragments become trapped.

Larry Goldstein agreed that mutant huntingtin causes pathology in the nucleus, which eventually leads to cell death. However, he added that a second mechanism by which huntingtin causes toxicity, and which may contribute to early

pathogenesis, is axonal blockage. Goldstein has observed that reducing the expression of wildtype huntingtin, or expressing proteins with expanded polyglutamine repeats, disrupts axonal transport in *Drosophila*. Furthermore, he has found that the levels of soluble motor proteins are decreased in both a *Drosophila* HD model and in R6/2 mice. Goldstein suggested that mutant huntingtin may cause axon blockage through the formation of aggregate clogs, as well as through the titration of motor proteins. This dual disruption could result in a lack of supplies reaching the synapse. Tobin wondered how the contribution of this disruption compares to observed transcriptional decreases in the levels of synaptic proteins.

Another deficiency in cellular movement appears to affect cytoplasmic mitochondria. Using tagged fluorescent proteins that are targeted to mitochondria coupled with live video microscopy, Ray Truant observed mitochondria crowding around the nucleus in cells expressing a mutated huntingtin fragment. Mitochondrial transport away from the nucleus was inhibited as revealed by a fluorescent “timer” protein which changes color over time allowing researchers to track the fates of proteins or, in this case, organelles. Applying ATP or forskolin restored mitochondrial movement, suggesting that mitochondrial movement was being inhibited by lack of ATP, but that mitochondria could produce that ATP when stimulated. Truant also observed that this phenotype, which includes abnormal mitochondrial morphology, was similar to that of dynamin-related protein-1 mutants. Indeed, when he crossed these mutants with HD mutants, the phenotype was accentuated. In addition, he observed that movement of huntingtin aggregates was also always towards the nucleus. Thus, the inhibition of transport away from the nucleus is likely to affect various organelles and/or proteins, particularly those associated with huntingtin.

Receptor trafficking is yet another process that may be disrupted by mutant huntingtin. Mannie Fan noted that previous studies from Lynn Raymond’s lab indicate that NMDA receptor currents are increased in the YAC model of HD, while overall NMDA receptor expression remains unchanged. Fan hypothesized that mutant huntingtin may increase NMDA receptor localization to synapses acting through the huntingtin-interacting protein HIP-1—an endocytic protein previously implicated in protein trafficking—and the actin cytoskeleton protein alpha-actinin. HIP-1 and alpha-actinin have putatively interacting domains. Co-immunoprecipitations and far-Western assays (Western assays that use a protein that is not an antibody as a probe) showed that, indeed, these interactions occur. HIP-1 appears to interact directly with alpha-actinin-4 and also interacts with the NR2B subunit of the NMDA receptor, which has been specifically implicated in striatal toxicity caused by mutant huntingtin. In addition, Fan observed that NMDA receptor NR1 subunits are increased at the surface of mutant cells. To confirm and extend these studies, she is now performing chymotrypsin-based assays to distinguish surface from internal receptor subunits. She is also conducting surface biotinylation experiments to examine whether NMDA receptor endocytosis, degradation, and/or insertion are affected.

Mitochondrial disruptions

Many studies have implicated mitochondrial disruptions and alterations in energy metabolism in HD pathology. Flint Beal, for example, noted that his group has used a number of techniques, including biochemical assays and imaging methods to measure energy-related metabolites, which have revealed that mitochondrial dysfunction may be an important pathological mechanism in HD. Consistent with these findings, administration of co-enzyme Q10, an electron acceptor essential to the function of mitochondrial complexes I and II, ameliorates HD symptoms in R6/2 mice. A clinical trial has so far shown only modest effects in humans, but it is expected that higher doses may be more effective.

Examining huntingtin’s link to mitochondria, Mathieu Lesort described localizing huntingtin to the outer mitochondrial membrane using subcellular fractionation, Western blot analysis and limited trypsin digestions. He also reported that mitochondria incubated in the presence of a mutant fragment of huntingtin or from a knock-in HD model had a reduced calcium threshold for opening of the mitochondrial permeability transition (MPT) pore and concomitant release of cytochrome c, an effect that could be abolished by cyclosporine A and ATP.

Monitoring ATP and ADP levels in HD knock-in mice with HPLC, McDonald reported that the cortex and striatum have decreased ATP/ADP ratios at early stages of disease. Moreover, the ratios correlate well with CAG length in human lymphoblastoid cells, suggesting they may be good biomarkers of disease. McDonald also found that both ADP and ATP levels are low in HD mitochondria, and glycolysis seems to be increased, perhaps to compensate for the mitochondrial dysfunction. The NMDA receptor inhibitor MK801 and the calcium chelator EGTA partially normalized mitochondrial ATP synthesis, suggesting the disruption may be linked, at least to some degree, to enhanced calcium influx through NMDA receptors.

Alterations in calcium signaling

An intriguing model of huntingtin toxicity which integrates various cellular components involved in calcium signaling, including NMDA receptors and mitochondria, was proposed by Ilya Bezprozvanny. Bezprozvanny recently discovered that the intracellular calcium release channel type 1 inositol (1,4,5)-triphosphate receptor (InsP₃R1) forms a complex with huntingtin and the huntingtin associated protein-1A (HAP-1A). Moreover, mutant huntingtin sensitizes InsP₃R1 to activation by InsP₃ *in vitro*. Bezprozvanny also observed that cultured striatal, but not hippocampal, neurons from the YAC128Q model of HD are sensitized to glutamate-induced apoptosis. Blockers of mGluR1/5 receptors which mediate calcium release through InsP₃R1s, the MPT pore, caspases –9 and –3, or NMDA receptors—in particular receptors containing the NR2B subunit which has been implicated in HD-associated striatal vulnerability—abolished the increased sensitivity.

Putting these data together, Bezprozvanny proposes that in HD cells, glutamate raises intracellular calcium levels abnormally through mGluR1/5 receptors and NR2B-containing NMDA receptors. The excessive calcium is initially buffered by mitochondria, but eventually, mitochondria saturate leading to the activation of calpain and caspases which results in huntingtin proteolysis and additional toxicity. The relatively high buffering capacity of mitochondria could explain HD's delayed onset. Bezprozvanny is now examining whether calcium signaling blockers may help ameliorate HD pathology.

The importance of context

Several participants presented data indicating that intracellular and extracellular context play key roles in determining the toxicity of mutant huntingtin. Jocelyne Caboche, for example, described how the striatum's dense dopaminergic innervation may significantly contribute to this region's vulnerability in HD. Caboche has shown that low doses of dopamine applied to primary striatal cell cultures act synergistically with mutant huntingtin to activate the pro-apoptotic JNK pathway and induce aggregate formation. Potentiation of c-Jun activation and concomitant cell death occurs via the production of reactive oxygen species (ROS). Using selective blockers and agonists of dopamine receptors, Caboche has further determined that these effects, as well as dopamine's potentiation of aggregation, are mediated through D2 receptors.

Cell-cell interactions also seem to be key in determining HD pathology in the cortex, as reported by William Yang. Yang and his colleagues used the Cre/LoxP system to generate mice that express mutant Huntingtin in either all neurons in the brain (pan-neuronal model), or only in the vulnerable cortical projection neurons (cortical model). Phenotypic analyses of these models revealed pathological cell-cell interactions are critical to cortical pathogenesis in HD. Other cell types in addition to neurons may also contribute to HD neuronal pathology. Olson proposed that glia may also contribute to pathology, noting that glia act as a source of cholesterol to make synapses, and cholesterol synthesis appears to be disrupted in HD as reported by Valenza.

In addition, Paolo Guidetti reported that microglial cells may importantly contribute to HD pathology. Guidetti has observed that the excitotoxin quinolinic acid and its bioprecursor, 3-hydroxykynurenine (3-HK), both produced by microglia, are elevated in the neostriatum and neocortex of HD patients at early stages of disease. In addition, he has recently evaluated the levels of these metabolites in three mouse models of HD: R6/2, *Hdh*^{Q92} and *Hdh*^{Q111}, and YAC128. Although the results varied between models, all showed elevated levels of one or both metabolites in the striatum and cortex, with no changes in the hippocampus. The results are consistent with the hypothesis that quinolinic acid plays a role in HD pathology and suggest that 3-HK, which potentiates quinolinic toxicity in the striatum, may also be important. Indeed, a genetic screen carried out by Flaviano Giorgini to identify loss-of-function suppressors of mutant huntingtin toxicity identified kynurenine 3-hydroxylase, the enzyme that generates 3-HK.

In addition to extracellular factors, the intracellular environment seems to play a key role in determining mutant huntingtin's effects. To examine the influence of different subcellular environments on polyglutamine aggregation, Anne Bertolotti and her colleagues targeted mutant huntingtin fragments that aggregate readily in the nucleus and cytoplasm to the endoplasmic reticulum (ER) and to mitochondria. Even at high expression levels, the fragments did not aggregate in either of these organelles as assessed by immunostaining, Western blots, and filter retardation assays. Once the mutant fragments were retrotranslocated out of the ER into the cytoplasm—because the ER lacks proteasomes, proteins targeted for degradation are retro-translocated into the cytoplasm—the fragments recovered their ability to aggregate, particularly when proteasome function was blocked to allow fragment accumulation in the cytosol. Thus, the results indicate that localized co-factors act to promote or prevent aggregation in a subcellular-specific manner.

Making sense of it all

Faced with such a large array of possible mechanisms to explain mutant huntingtin toxicity, Leslie Thompson encouraged participants to discuss how they would begin to make sense of it all: What evidence should be required to connect a mechanism to HD pathology? What are appropriate readouts? How much does a mechanism have to alter a particular phenotype to be considered significant? What constitutes *in vivo* relevance? What mechanisms can be ruled in or ruled out as fundamental to the disease process? Which ones will be of most therapeutic value? Which ones are primary mechanisms and which are secondary? Does it matter, if they have therapeutic value?

Thompson proposed a branching pathway to explain HD pathology, modifying a model originally presented by McDonald in which the CAG expansion triggers a cascade of events that culminate in HD. The process is complex because, as noted by Jang-Ho Cha, numerous mechanisms with downstream initiating events are probably involved. In addition, many mechanisms are not clearly linked to neuronal death, and their pathogenic relevance is difficult to assess. And as pointed out by David Rubinsztein, there may be several parallel pathways that are disconnected from each other early on. On the one hand, it will be important to discern which alterations truly underlie the disease process and which ones are epiphenomena or compensatory changes, as stressed by McDonald. On the other, it will be important to dissect the causal links between relevant alterations.

Despite the many complications, participants were optimistic about the future. Cha, for example, noted that the multiplicity of mechanisms, although daunting, suggests the existence of multiple therapeutic targets. He also pointed out that neuronal dysfunction, although more difficult to monitor compared to cell death, may be easier to correct. And the slow onset of HD, although difficult to study experimentally, offers a potentially large therapeutic window. Although some participants considered that specific targets for drug development were lacking, others were impressed by the emerging possibilities (see “Discovery of therapeutics for HD” below). Hayden, for example, pointed out that early alterations in calcium regulation, which have been confirmed by several labs, may prove particularly amenable to therapeutic interventions (e.g., proteases, NR2B receptors, IP₃Rs). And Anne Graybiel was excited about the emerging integration of diverse findings at the molecular, cellular and systems levels.

To capitalize on these promising developments, participants offered various suggestions. Thompson, for example, proposed genetics could provide a gold standard for linking candidate mechanisms to HD pathology. If a pathway alleviates the HD phenotype when it is genetically knocked out, then it likely plays a role in the disease process. Rubinsztein added that showing graded effects with genetic dosage could serve as a useful benchmark, and Fishbeck suggested that dependence on CAG repeat length would also be a valuable criterion. Developing a treatment that can be tested in patients was proposed as another gold standard for evaluating pathogenic mechanisms. As an example, Beal noted his group’s investigation of mitochondrial dysfunction and the concomitant use of co-enzyme Q10 to try to reverse HD pathology.

The use of appropriate controls was also discussed. Rubinsztein stressed the importance of making valid comparisons when working with transgenic animals. As an example, he praised Graham’s efforts to match the expression levels of mutant huntingtin in experimental and control YAC transgenics. Illustrating the importance of examining alternative interpretations, Aaron Bowman showed that reporter systems do not always report what they are designed to. Using a reporter construct for assessing the functionality of the ubiquitin proteasome system (UPS) in a model of the polyglutamine disorder spinocerebellar ataxia type 7 (SCA7), Bowman observed that the formation of aggregates was inversely correlated with the UPS reporter signal. Usually, increased reporter levels reflect decreased UPS function. A careful examination of the data revealed, however, that the reporter signal was being affected by transcriptional alterations caused by the disease. Bowman concluded that transcriptional dysregulation, not UPS dysfunction, correlates with SCA7 pathology.

Several areas of research were highlighted as particularly promising for future exploration. Goldowitz and Rubinsztein underscored the potential of genetic modifier studies. Indeed, exciting advances in this area were reported in a variety of model systems, including yeast, *C. elegans*, *Drosophila*, knock-in mice, and humans in the poster sessions. Goldowitz also considered that experimental approaches in which competitive interactions at the tissue, cell and molecular levels are set up would be particularly useful for dissecting causal links. In addition, he predicted that bioinformatics analyses applied to gene expression data and protein-protein interactions will continue to provide important insights. Michael Levine reminded participants that the function of neurons is to make circuits and, consequently, more investigation into the electrophysiological disruptions caused by HD was warranted. And David Lowe added that further work was needed in identifying and characterizing cognitive alterations.

Discovery of therapeutics for HD

Although much remains to be understood about the mechanisms of HD pathology, a wide range of efforts towards developing therapeutic agents was discussed at the meeting. Participants described strategies for identifying candidates ranging from high throughput screens of large libraries of compounds, to the rational design of molecules aimed at reversing specific HD alterations. They presented data on candidates ranging from those still at the discovery stage, to those undergoing pre-clinical testing. The target specificities of the candidates also spanned a broad range, including agents directed at blocking very early events leading to HD pathology, to compounds directed at ameliorating HD-associated symptoms.

High throughput screens

Illustrating the potential of small molecule screening approaches, Aleksey Kazantsev and Phoebe Harjes reported the identification of therapeutic candidates using high throughput assays that monitor polyglutamine aggregation. Kazantsev and collaborators used a yeast-based assay in which both growth and aggregate formation were monitored, while Harjes and colleagues relied on an *in vitro* assay to assess aggregation of a GST fusion protein using filter retardation assays and imaging of immunostained aggregates. Kazantsev's group initially screened 16,000 compounds obtaining 9 hits which were then tested in PC12 cells, followed by testing in COS cells using filter retardation assays. When subsequently tested in brain slice cultures from R6/2 mice, however, the compounds were not as effective and some were toxic. Screening a library of close-analogues to find improved alternatives, the researchers homed in on a high potency compound that is effective in PC12-based assays, R6/2 brain slice assays, and *Drosophila* toxicity assays. Kazantsev and colleagues now plan to go through an additional cycle of medicinal chemistry optimization before testing the compound in mice. In addition, they will analyze 16 new hits resulting from re-screening the original 16,000 compounds using a modified version of their primary yeast assay.

Harjes and her colleagues in Erich Wanker's lab have also identified promising candidates. Based on an initial screen of approximately 184,000 compounds, Volker Heiser identified benzothiazoles as dose-dependent inhibitors of polyglutamine aggregation. Immunoblotting, electron microscopy and mass spectrometry were used to confirm the finding, and now the group has begun testing the compounds' effects *in vivo*. The data indicate benzothiazoles can reduce aggregation in PC12 cells expressing an inducible mutant huntingtin construct. A particularly potent derivative, PGL-034, reduced aggregation by 20% in cultured cells. Preliminary results indicate PGL-034 is able to restore the climbing activity that normally deteriorates in a *Drosophila* model of HD. Another derivative which inhibits aggregation very effectively is currently under investigation.

Pre-clinical studies

Emma Hockly and colleagues are performing pre-clinical studies on other benzothiazole derivatives. Disappointingly, however, riluzole, a benzothiazole which reaches high concentrations in blood and brain through oral administration, failed to improve rotarod performance, grip strength, exploratory activity, weight loss, circadian rhythm disruption, and aggregate pathology in R6/2 mice. Another benzothiazole derivative identified by Heiser, PGL-135, reached only very low concentrations in R6/2 mice because it is metabolized quickly.

Additional compounds in pre-clinical trials include ethyl eicosapentanoic acid (EPA), an omega-3 fatty acid which has been tried unsuccessfully as Lax-101 in humans, cystamine, a transglutaminase inhibitor that also inhibits caspases and increases antioxidant levels, and BDNF. Using the YAC128 model of HD, Blair Leavitt and colleagues have used quantitative neuropathology as a primary outcome measure and behavioral endpoints as well as cell counts, as secondary outcomes to assess the therapeutic potential of these compounds. To date, EPA has shown no significant neuroprotection nor improvement in the levels of the medium spiny neuron marker DARPP-32, and only a mild improvement in rotarod performance and hypoactivity. Cystamine administration, on the other hand, resulted in decreased striatal atrophy and decreased loss of striatal neurons, but without behavioral nor DARPP-32 improvements. Even with early cystamine treatment, at 4 months of age, behavioral outcomes remained unchanged. No significant effects have been detected as of yet using BDNF.

HDAC inhibitors: Lessons in therapy timing and the potential of combination therapies

An important question highlighted by these trials is at what stage, or stages, in the disease is it advisable to test different therapeutic candidates, particularly since the development of HD is such a protracted process. Early administration usually provides a greater chance of observing beneficial effects, yet later testing offers the opportunity of assessing whether candidates might be helpful for post-symptomatic patients. To examine this question for one therapeutic candidate, the HDAC inhibitor phenylbutyrate, Sue Brown and colleagues injected the compound into N171-82Q mice after symptom onset, in contrast to previous studies which had examined its effects before. Although no improvements were observed in motor performance, weight loss, or the presence of nuclear aggregates, there was a 23% increase in lifespan and decreased cerebral and neuronal atrophy. In addition, microarray analysis of the striatum indicated that some transcripts encoding potentially beneficial proteins—components of the UPS, for example—were increased, while transcripts encoding potentially detrimental proteins—caspase-9, for example—were decreased.

Another study of the efficacy of HDAC inhibitors underscored the potential benefits of using combination therapies. To determine which HDACs are involved in mediating mutant huntingtin toxicity in *Drosophila*, Marsh genetically reduced each of the three different classes of HDACs and found that decreasing classes I and III, but not II, was effective in alleviating HD toxicity. Interestingly, the simultaneous reduction in classes I and III resulted in a synergistic effect which was reproduced using class-specific pharmacological inhibitors. Thus, it is possible that therapies could be developed in which two or more inhibitors are used simultaneously at low doses to achieve better clinical outcomes, while minimizing undesirable secondary effects. In addition, these combination cocktails could include other compounds besides HDAC inhibitors. Several participants, including Beal and Vernon Ingram, noted that combination therapies would probably be of great value and should be pursued more vigorously.

Intrabodies

Seeking to curb HD pathology at an early stage, some researchers are developing intrabodies—single chain Fv antibody fragments—to either alter the kinetics of mutant huntingtin misfolding or interfere with mutant huntingtin's access to other cellular components. Intrabodies are powerful reagents because they have the conformation-specific selectivity of antibodies, yet can be readily subjected to genetic engineering to improve their stability and affinity. Messer presented an update of these efforts, including data from three different laboratories. She noted that HD-relevant intrabodies have been either selected from intrabody phage display libraries, or derived from known anti-huntingtin monoclonal antibodies. To test their effectiveness, researchers are using assays in mouse brain slices and in *Drosophila*. An important control required in all assays is the use of a non-specific intrabody to rule out a decrease in mutant huntingtin translation resulting from the high expression levels of the intrabody.

So far, intrabodies directed against expanded polyglutamine have exacerbated both aggregation and toxicity. But intrabodies that flank the polyglutamine region—including C4 which recognizes the first 17 amino acids of mutant huntingtin and MW7 which recognizes the polyproline stretch—have proved particularly effective at decreasing aggregation and toxicity. C4, which is very stable, was identified by Messer's group from a phage display library. In a *Drosophila* HD model, C4 had a dramatic effect on rescuing pupal emergence (a measure of neural intactness), decreased aggregate formation, increased survival, and decreased photoreceptor degeneration. The MW7 intrabody, derived from a monoclonal antibody generated by Patterson, reduced cell death and aggregation as assessed by an organotypic slice assay developed by Peter Reinhart and Don Lo.

But several challenges lie ahead. Although promising, intrabody effects have so far been partial. To address this problem, several intrabodies are now being taken through multiple rounds of re-engineering. In one case, an intrabody's binding was improved from a 5:1 ratio to a 1:1 ratio after 12 rounds of re-engineering in Ingram's and Dane Wittrup's labs. Messer noted that further engineering will be applied to both C4 and MW7. Another challenge is delivery. Messer's group has used the equine lentivirus EIAV to deliver the C4 gene to R6/1 neurons with some success, but additional work is needed.

Messer estimated that preclinical studies would take 18 to 24 months after the re-engineering process, and noted that each round of mutagenesis and selection takes about 2 months. Use of the *Drosophila* system as the primary test assay should help optimize these efforts.

Messer also described several areas for future exploration. The use of C4 and MW7 intrabodies jointly, for example, may be particularly powerful helping prevent beta-folding from either end of mutant huntingtin. Moreover, intrabodies directed against huntingtin cleavage sites or HD modifiers may have therapeutic value, as well as combining intrabodies with other therapies.

RNAi

Attacking HD even more closely to its primary source, Scott Harper, Stefan Kochanek, Edgardo Rodriguez, and Gustavo Tiscornia reported their experiences developing RNA interference (RNAi) reagents to suppress mutant huntingtin expression. As noted by Beverly Davidson and Rodriguez, RNAi offers a tool for advancing basic research, as well as the potential for developing therapeutic candidates. Because of its primary effects on HD, it has the potential of illuminating the contributions of mutant huntingtin's gain- and loss-of-function effects, and helping distinguish primary from secondary effects. In addition, it offers a potentially large window of therapeutic efficacy.

Harper and colleagues identified small hairpin RNAs (shRNA) targeting huntingtin exon 2 and 3 that significantly reduce wildtype and mutant huntingtin *in vitro*. The group had originally intended to target huntingtin exon 1, but they were unable to find an effective shRNA in this region. Harper cloned the effective shRNAs into adeno-associated virus type 1 (AAV1)—a neurotrophic, nonpathogenic parvovirus which yields high-titer, stable infections—for delivery into cells. Experiments in cultured cells yielded positive results as assessed by quantitative RT-PCR and Western blot analysis. More importantly, injection of the viruses into the striata of HD mice (N171-82Q) resulted in a 55% reduction in huntingtin expression, as well as improved rotarod performance and stride length at 18 weeks post-infection.

Rodriguez and colleagues, on the other hand, identified a shRNA upstream of huntingtin's ATG site, siHUNT, as particularly effective at knocking down huntingtin expression. Using rAAV5 for delivery, they injected R6/1 mice just before nuclear inclusions become visible, at 6 to 8 weeks of age. By 16 to 18 weeks, Rodriguez detected an 80% reduction of huntingtin RNA as assessed by quantitative RT-PCR. Immunohistochemistry also revealed a qualitative decrease in huntingtin protein although, surprisingly, Western blots failed to reveal a significant reduction. Rodriguez also observed moderate amelioration of pathology, including an increase in enkephalin and DARPP-32 transcript levels, and a delay in the development of the clasping phenotype. Other behavioral phenotypes, however, remained unchanged.

Optimizing vector function has also proved challenging. As noted by Tiscornia, the efficiency of lentivirus vectors depends on the sequence of the elements it carries such that different combinations must be tested. However, even when the sequence has been optimized *in vitro*, the vector may fail *in vivo*. For example, Tiscornia generated a line of YAC72 HD mice that express a huntingtin shRNA but observed no decrease in huntingtin levels. Another construct carrying three copies of DNA encoding a shRNA yielded no detectable levels of shRNA. One possibility is that the construct caused silencing of polymerase III promoters. Another one of Tiscornia's constructs, L29, appears to have variable effects when injected into YAC72 striata, with some evidence of it causing neurodegeneration. Tiscornia is now examining whether the vector is toxic.

Yet another challenge associated with siRNA therapies is specificity. Aronin noted that the extent to which shRNAs have off-target effects is variable and difficult to predict. In some cases, effects have been reported on sequences that are up to 50% mismatched. Tiscornia, for example, designed human-specific and mouse-specific shRNAs to test in YAC72 mice which carry a full-length copy of human huntingtin in addition to their endogenous mouse alleles. The shRNA designed to be human-specific, however, also knocked down the endogenous mouse alleles, even though the sequences differed by six nucleotides.

Such lack of specificity may be particularly problematic for approaches seeking to knock down mutant, but not wildtype, huntingtin by targeting single nucleotide polymorphisms (SNPs) linked to the huntingtin mutant allele. Such custom-made repressors will require individualized off-target testing. As noted by Davidson, the costs could be prohibitive. On the other hand, she added, it is unclear how critical it will be to avoid knocking down wildtype huntingtin. Perhaps partial reductions in mutant and wildtype huntingtin levels may be sufficient to ameliorate HD pathology while preserving huntingtin's normal function. It will be interesting to know if the partial effects that have been observed are due to uniform low levels of suppression, or to a subset of cells with high levels of suppression.

One way to circumvent the problem of specifically targeting mutant huntingtin is to deliver a shRNA-resistant copy of wildtype huntingtin along with the therapeutic shRNA. To pursue this option, Kochanek and colleagues are using "gutless" adenovirus vectors which lack parts of the viral genome and can thus carry large amounts of heterologous DNA. Using a first generation of these vectors, they have transfected cultured cells with DNA encoding shRNA directed against the ATG region of huntingtin, and DNA encoding huntingtin exon 1 fused to eGFP. The researchers observed a decrease in endogenous huntingtin levels and a reduction in aggregate formation.

The researchers are also working on improving delivery specificity. Adenoviral vectors transfect glia more readily than neurons, so Kochanek and colleagues are introducing cysteines into the viral capsid protein to covalently link cell-specific antigens through the cysteines' thiol groups. For example, linking transferrin to the capsid protein enabled the

specific transfection of cells expressing high levels of transferrin receptors. Kochanek is now searching for neuron-specific targets to ultimately deliver shRNAs that target huntingtin mRNA. Additional delivery options are being tested by other groups, including the use of lentivirus vectors, as described by Tiscornia, and chemically modifying shRNAs to make them membrane-permeable, as noted by Neil Aronin.

Participants also commented on the design of RNA suppressors. Davidson pointed out that the “rules” commonly cited for designing shRNAs do not always hold and she urged participants to avoid being too constrained by these recommendations. Aronin added that there are other ways to repress translation in addition to shRNAs, such as micro RNAs. He also noted that single-strand RNAs sometimes work better than double-strand RNAs. Several biotechnology companies are examining these options which may prove beneficial to HD research.

RNAi's future in clinical trials was also discussed. Davidson noted that setting up tests in primates will probably be important, as well as obtaining input from clinicians. She suggested setting up a workshop to discuss these issues. Ron Mandel and Rodriguez pointed out the potential risks and ethical considerations of injecting viruses into patients. Mandel noted that several clinical trials are currently underway using AAV vectors, but he thought the associated risks had not been carefully considered. He and Rodriguez deemed that introducing viruses that cannot be turned off is, at this time, unethical. The half-life of shRNAs and the activity of their promoters *in vivo* are not well understood, and could last indefinitely. Davidson noted that the use of inducible promoters that can be switched off may help minimize this problem in the future, but a panel of ethicists, biologists, and physicians will probably be necessary to examine this question in depth.

HD-associated symptoms

Although an ideal HD therapy would block HD pathology at its root, the amelioration of disease-associated symptoms may benefit patients greatly. In addition, when this amelioration can be achieved with compounds that have been previously tested, the benefits may be reaped very quickly. For example, Kathleen Clarence-Smith and colleagues from Prestwick Pharmaceuticals, Inc. are targeting HD choreic symptoms using tetrabenazine, a depletor of monoamines in nerve terminals that appears to ameliorate chorea much better than any other drug currently approved in the United States. Indeed, HD patients are already benefiting from its use in several European countries. Tetrabenazine has a high selectivity for the vesicular monoamine transporter hVMAT2, which is predominantly expressed in striatal dopaminergic terminals. This is in contrast to other monoamine depletors, such as reserpine, which binds to both hVMAT1 and hVMAT2, and consequently also affects blood pressure and gut mobility. Indeed, HD patients often stop taking anti-choreic medications because of their unpleasant side-effects.

To more carefully assess tetrabenazine's potential as an anti-choreic medication for HD, Clarence-Smith is collaborating with the Huntington Study Group to conduct the largest tetrabenazine study ever, including clinical trials in 16 sites across the United States. Extending previous, smaller-scale studies, the trials indicate that tetrabenazine dramatically ameliorates HD chorea, and that the beneficial effects are long-lasting. Tetrabenazine does not lose its potency or efficacy even after years of treatment, in contrast to other anti-choreic medications. Although tetrabenazine can have secondary effects, these can be effectively controlled by lowering patients' doses. One complicating factor discovered by Clarence-Smith is that there is great variability between individuals' dose requirements—production of tetrabenazine metabolites is very heterogeneous, and various VMAT isoforms exist. As noted by David Lowe, however, this response variability, often linked to single nucleotide polymorphisms, is common to many compounds and is now being incorporated into the design and evaluation of clinical trials.

In sum, participants were enthusiastic about tetrabenazine's prospects for treating HD chorea. Prestwick is hoping to submit a New Drug Application (NDA) in the near future, and if the FDA approval process is expedited, tetrabenazine could benefit U.S. patients reasonably soon.

Another study of HD symptoms was presented by Jenny Morton. Following up on observations in humans suggesting that the circadian rhythms of HD patients are abnormal, Morton examined the activity patterns of R6/2 and control mice. Unlike normal mice which have high levels of activity during the night and low levels during the day, the R6/2 animals had scattered bursts of activity throughout the day. By 14 to 16 weeks of age, their circadian patterns were completely disrupted. In addition, *in situ* hybridizations revealed that the levels of several transcripts involved in driving the circadian clock were altered.

Bruce Kristal discussed another HD-associated pathology that may be ameliorated by known compounds: the induction of the mitochondrial permeability transition (MPT). MPT is a disruption in mitochondrial function associated with cell death, calcium dysregulation, abnormal caspase activity, and impaired energetics. Kristal and colleagues have been conducting screens for MPT inhibitors in an effort to find compounds for treating neurological disorders, in particular stroke.

One class of molecules they've identified are moderately active compounds with tricyclic organic rings, including several FDA-approved drugs such as anti-depressants, anti-psychotics, and anti-histamines. The compounds have minimal effects on normal mitochondrial physiology, cross the blood-brain barrier, and have a long safety record. Using informatics analyses, Kristal and co-workers have narrowed their focus to 4 lead compounds. One of these, promethazine, resulted in over 50% reduction in stroke volume in mice. Interestingly, as pointed out by Beal, promethazine has also been shown to reduce damage in the 3-NP-induced model of HD. However, Bezprozvanny noted that MPT occurs very late in the HD cascade of alterations, so affected cells may be too sick at that point to benefit from its inhibition. Kristal noted that individual cells harbor many mitochondria and rescuing some with early damage may facilitate cell recovery. He also reiterated that a MPT inhibitor would probably not cure HD, but may mitigate some of HD's secondary pathology.

Optimizing flow through the drug discovery pipeline

Key to accelerating the development of these therapeutic candidates will be automating, standardizing, and refining the assays to test them. As noted by Wittrup and Reinhart, quantitative pharmacology will be needed, as well as standardized assays, models, delivery systems, and payloads. Reinhart considered that target identification and validation were slowing down the movement of therapies into the clinic. He urged participants to invest more effort on developing and standardizing pre-clinical animal models. One way in which the Hereditary Disease Foundation and other funding agencies could help establish these standards, Wittrup proposed, is by limiting funding to those who agree to follow particular guidelines.

Addressing the issue of pharmacokinetics in animal models, Philip Lowden and colleagues are using analytical chemistry techniques to monitor the levels of various drugs in mice. Their goal is to help identify optimal dosages and routes of administration to achieve effective drug concentrations in the brain and maintain them without inducing toxicity.

The need for high throughput, automated behavioral tests is also rapidly growing as the number and size of pre-clinical and clinical trials increase. Addressing this problem, Miriam Hickey and colleagues have evaluated the effectiveness of automated open field, climbing, and running wheel tests using the R6/2 and Q140 knock-in mouse models of HD. Their data indicate that the tests enable the early, quantitative, and high throughput detection of behavioral abnormalities in different HD models prior to overt phenotypes such as weight loss, rotarod deficits and grip strength alterations.

Participants also discussed how to best direct future efforts to develop HD therapies. Some participants favored focusing on targeting HD's primary alterations and increasing the selectivity of target explorations. Tobin, for example, stressed the importance of developing ways to prune down the number of hypotheses to be tested. Reinhart, on the other hand, favored a parallel approach in which many candidate therapies are funneled through animal model testing. Gusella cautioned, however, that it will be important to understand, at least to some extent, the position of secondary targets in the HD pathological cascade(s) to ensure the use of appropriate assays to monitor their effects before pushing them too far through the pipeline. Another consideration put forth by Morton is that there are multiple clinical targets: there are patients who have not been born yet, and there are symptomatic patients whose health is steadily declining now. Consequently, it will probably be fruitful to invest in developing various therapies, including ones that act pre-symptomatically and ones that act symptomatically, as well as ones that are in the early stages of development and those that are more mature in their development for human use.

The topic of how much basic knowledge is necessary for therapy development surfaced repeatedly. Some participants such as Peter Lansbury, noted that the mechanisms of action of most drugs on the market today are poorly understood, and predicted that much clinical progress could be made without necessarily obtaining much additional basic knowledge. Indeed, Marlene Haffner, Director of the Office of Orphan Products Development at the FDA praised participants for their progress, but urged them to move more quickly into clinical trials. She noted that not only could therapeutic candidates move into clinical trials with little mechanistic information, but that some pre-clinical analyses, such as dosage examination, could be minimized. Encouraging participants to translate their basic findings into clinical advances, Haffner noted that her division awards small bench-to-bedside grants. Morton agreed that if a candidate therapy works it should be pursued vigorously, even if its mechanism is uncertain, but she also emphasized that obtaining more mechanistic information would greatly enhance the community's chances of identifying effective therapies.

To help researchers prioritize interventions for testing in pre-clinical and clinical trials, Bernard Ravina and colleagues are systematically compiling clinically relevant HD data involving compounds that are currently approved by the FDA (Systematic Evaluation of Treatments for HD, SET-HD). Modeled on a Parkinson's disease initiative, the project has identified 190 interventions based on literature searches and nominations from experts and lay people. Using a hierarchical approach, the researchers generated a list of approximately 40 top candidates for testing in clinical trials. Evaluation criteria

included: rationale for use in HD, consistent preclinical efficacy data, kinetics and blood-brain barrier penetration, and prior use and safety in humans. Ravina urged participants to examine the database at www.huntingtonproject.org and provide suggestions and comments.

Animal models

Participants also discussed the future use and development of animal model systems. David Housman was excited about the many new insights into human disease recently provided by mice and the striking concordance of lower eukaryotes and mice. He noted, however, that to obtain robust data it will be essential to test the roles of different genes and pathways in multiple models. It will also be important, as noted by Steve Dunnett, to optimize the selection of models for particular research aims—rat models, for example, may be particularly well-suited for probing the electrophysiological underpinnings of HD, while primate models may be necessary for evaluating transplant therapies. Gillian Bates added that it is still unclear which models will prove most useful at reproducing human HD, so it is best to avoid excluding models at this stage. Strengthening the connections between the models and human HD will be critical, as noted by Carl Johnson and Jim Gusella, but it will be a complicated process given that it is still uncertain what exactly constitutes the human phenotype; which pathologies are primary and which are epiphenomena.

To optimize the use of available models, Dunnett, Levine, and Bates directed participants to the Hereditary Disease Foundation's website for guidelines drawn up at a workshop in Cardiff, U.K. for the standardization of protocols and readouts (http://hdfoundation.org/workshop/200207Report.htm#_ednref3). For example, the guidelines recommend using two different model systems, one with a rapid onset and progression of disease (a fragment model, e.g.) and one with a slower onset and progression (a full-length model, e.g.) to test candidate therapeutic compounds. Reproducing results in at least two different labs is also recommended. A description of standards for performing behavioral tests is also available at the site. As noted by Bates, the Cardiff meeting was not intended to impose rules, but to exchange ideas and generate an informal list of evolving recommendations. Dunnett suggested setting up a recurrent review group to keep the recommendations current. Dealing with different strain backgrounds and the development of cognitive assessments were noted as two areas that would particularly benefit from such a review meeting.

Gusella emphasized the importance of mouse models, noting that beyond enabling the testing of drug candidates for future human use, they can yield valuable information about compounds that provide insights about the disease process and how to block it, although they may never be appropriate for human use. In addition, Gusella considered that it will even be useful to test compounds that have already been approved for humans in animals to continue enriching the compendium of animal data.

Despite the wealth of current HD models, Larry Goldstein considered that still better ones are needed. He emphasized that a detailed understanding of the timing of pathology will probably be essential for prioritizing candidate therapies. Generating new models using embryonic stem cells, he proposed, may help achieve this goal. Yang pointed out that powerful new tools for doing mouse genetics, such as bacterial artificial chromosome (BAC) libraries and the possibility of generating large-scale knock-out collections, also promise to advance HD research substantially. He noted that in addition to using mice for conducting drug screens, genetic interaction experiments can be performed by crossing different transgenic mice. Yang's own experiments using the Cre/Lox system to generate pan-neuronal and cortical models of HD illustrate some of the powerful capabilities of mouse genetics.

In addition, more detailed characterizations of some recently developed models are needed. Simon Brooks, for example, presented a longitudinal study of motor deficits in knock-in CHL1 (carrying 80 CAG repeats) and CHL2 (carrying 150 CAG repeats) mouse lines. Compared to R6/2 mice, motor deficiencies were mild and there was much variability within groups, both in the nature and severity of the deficits. Nevertheless, a progressive decline in motor skills was observed and differences between CHL1 and CHL2 were detected.

Rat models are gaining popularity as well, as noted by Carl Johnson and David Lowe. Reinhart and Lo have developed a biolistics-based model of HD in rat striatal slices, Nicole Deglon has generated a rat lentiviral model of HD, and von Hörsten has created transgenic rats carrying a huntingtin fragment with 51 CAG repeats. These models are particularly valuable because many experimental manipulations—for example, electrophysiological recordings and cannula implantations—have been optimized for rats. In addition, rats may be useful for pharmacokinetic studies because of their slower metabolic rate compared to that of mice.

Clinical trials

Participants also discussed strategies to optimize clinical trials. Several participants noted that the traditional sequence of clinical trial phases is often less than optimal. For example, Sir Michael Rawlins considered that merging phases I through III could lower costs and accelerate the process substantially. Haffner noted that, indeed, she has observed a trend towards compressing these phases. The degree of compression, of course, depends on the particular therapeutic candidate. Rawlins also favored continuing treatments while statistical analyses are underway, and urged participants to be more creative in their trial designs. He considered that Bayesian approaches to data analysis would be particularly fruitful, although David Lowe noted that a consensus on the application of these statistical tools to clinical trials has yet to be reached. Olson suggested recruiting cancer statisticians who have ample experience with complex data analysis. The use of adaptive trial design, i.e., modifying ongoing trials based on accrued data, was also discussed. Ira Shoulson noted that his group is examining various options for applying this approach. Bias can be introduced when unblinding a study, but with proper controls, it affords several advantages. For example, it can help recognize ineffective candidate therapies more quickly, so they can be terminated, minimizing costs and effort.

Lowe, who has extensive experience in the pharmaceutical industry, agreed with Rawlins that clinical trials need to be streamlined. He also added that the pharmaceutical viewpoint has to be strengthened in HD. Because of HD's relatively low incidence, small biotech companies and non-profit organizations will play a key role, he noted, compared to big pharmaceutical companies which prefer investing in very common diseases.

Several participants considered that studies on pharmacokinetics and toxicity should be started early. Lowe noted that between 50% and 60% of therapeutic candidates for central nervous system disorders run into problems with cardiovascular toxicity. Another participant added that because therapeutic agents will probably require chronic administration over many years, medicinal chemistry should be applied early on to minimize toxicity. Reinhart noted, however, that it will be important to avoid discarding compounds because of toxicity too early because they may provide valuable mechanistic insights. The chemistry required for drug synthesis and the cost of a therapy should also be addressed early, noted Lowe.

Powering trials to detect small improvements was also considered critical. As noted by Dunnett, there are many small, underpowered studies that are confusing the literature and encouraging patients to take compounds that complicate future clinical trials. Drawing from his experience as a pediatric oncologist, Olson noted that most of the improvements in childhood cancers have come in increments of 3-10%, which can only be detected in appropriately powered trials. Olson urged participants to increase patient enrollment in clinical trials, noting that 90% of all pediatric cancer patients are enrolled in trials at any given time. He also noted that current funding mechanisms for HD trials at the NINDS do not facilitate the testing of multiple compounds. Shoulson, who is the executive leader of the Huntington Study Group, completely agreed with Olson's recommendation for increased patient enrollment, noting that less than 5% of HD patients are currently enrolled in clinical trials. One limitation has been the recruitment of neurologists, but the HSG has now enlisted several hundred neurologists to participate in clinical trials. Thus, trial capacity should not be a limiting factor. Shoulson added that incremental gains in the development of HD therapies are already emerging and noted that safety/tolerability studies on minocycline and riluzole had just been published. He also pointed out that drugs with moderate effects should not be underestimated in their ability to focus efforts, particularly in industry, towards the development of better drugs. In addition, as suggested by recent results of the effects of co-enzyme Q10 on Parkinson's disease patients, altering the dosage of a compound with moderate effects may importantly enhance its efficacy.

The other major factor for powering clinical trials is the ability to monitor the disease process and predict phenoconversion. In theory, a drug that completely cured HD would require a tiny set of patients and a low level of predictability to reveal its effects. But the number of patients rises steeply when testing drugs with more moderate effects. So in order to keep patient numbers within a reasonable range, it is essential to obtain markers that allow quantitative and reliable monitoring over the course of the disease. In addition, presymptomatic biomarkers could enable early therapeutic intervention, as well as reveal mechanisms of pathology and new drug targets. Current methods to track the progression of HD based on neurological symptoms lack specificity, sensitivity and reproducibility, and do not allow early disease monitoring. Many participants, including David Lowe, Olson, and Haffner, considered that identifying biomarkers is a top priority in HD research.

Biomarkers

As noted by Shoulson, the HSG has designed two complementary observational studies (known as PHAROS and PREDICT-HD) to identify early signs of onset in at-risk HD populations. A particularly ambitious project known as COHORT involves collecting information from individuals who are affected, at risk, or members of an HD family to create a longitudinal database including neurological data, family history, and biological samples. In addition to providing a rich collection of data on HD, these studies are expected to contribute to the search for markers of HD progression.

In addition, promising biomarkers have already been identified in smaller-scale, basic research studies. Ross and colleagues, for example, analyzed the brains of presymptomatic patients using structural MRI imaging and found that striatal volumes begin to atrophy at least 10 years before the onset of motor symptoms and decline steadily during the presymptomatic stage. By the time of motor onset, striatal volumes are approximately half the normal size. In addition, alterations in subcortical white matter were revealed by decreased fractional anisotropy, in some cases even earlier than the striatal volume changes. Also, functional MRI using a Stroop-like interference task revealed changes in brain activity patterns that in some cases preceded striatal volume changes. Interestingly, the alteration in activity was evident even when the actual performance of the task was normal. The PREDICT-HD study will extend Ross's findings using a larger group of patients.

Promising biochemical markers are also emerging. Asa Petersen, for example, presented data indicating that the levels of the hormone orexin in cerebrospinal fluid (CSF) may provide a useful marker of HD progression. Petersen found that hypothalamic neurons, in particular the orexin-producing cells of the lateral hypothalamus, are progressively lost in R6/2 mice. Because orexins regulate sleep and their decrease is a diagnostic marker of narcolepsy, Petersen searched for signs of narcolepsy in R6/2 mice. Confirming her prediction, she found a progressive development of narcoleptic episodes that correlated with orexin loss, as assessed by behavioral observations, and EEG and EMG recordings. Petersen has also observed orexin loss and hypothalamic neuron atrophy in humans, and is now assessing the incidence of narcolepsy or other disturbed sleeping patterns in HD patients. Of particular interest for the search of biomarkers, orexin loss could be tracked in CSF in mice. Petersen is now assessing whether the same is true in humans.

Leptin was proposed as another possible biomarker for HD. Alterations in adipose tissue, such as wasting despite high caloric intake, have been described in HD. Based on such observations and the fact that adipose tissue is an endocrine organ that regulates energy metabolism which is known to be disrupted in HD, Karen Reue and her colleagues examined adipose tissue function in the R6/2 and Q140 knock-in mouse models of HD. In addition to observing alterations in fat pad mass, the researchers found disruptions in the expression of adipocyte-specific genes. In 4-week-old R6/2 mice, for example, the expression of mature adipocyte genes was increased, although fat mass was decreased. Q140 knock-in mice also showed expression abnormalities. In later stages of disease, Reue observed a repression of the expression of adipocyte-specific genes. Of particular relevance to biomarker identification, she noted an altered relationship between leptin levels and adipose tissue mass in both mouse models. Because leptin is easily quantified in small amounts of blood, it may provide a biomarker for HD.

Another potential source of HD biomarkers is muscle. As noted by Andy Strand, muscle is an accessible tissue whose physiology responds to metabolic, hormonal, and central nervous system inputs. Seeking to identify biomarkers to power clinical studies as well as to gain mechanistic insights, Strand and co-workers analyzed gene expression using microarrays in skeletal muscle of R6/2 mice. The activities of genes associated with fast-twitch muscle function—e.g., glycolysis—were decreased, while those involved in slow-twitch function—e.g., lipid catabolism—were increased. Very similar findings were obtained using human tissue. Strand hypothesizes that HD drives a transition from fast to slow muscle fiber types. Indeed, Northern blot analysis revealed complementary changes in alpha-actinin 2 and 3, consistent with this transition. There are several known causes of fast to slow fiber conversion, including aging, hypothyroidism, endurance training, chronic low frequency stimulation, and depletion of creatine and/or ATP. The chorea of HD may mimic chronic low frequency stimulation, and HD is known to cause alterations in creatine and ATP levels. Indeed, as previously mentioned, McDonald's studies suggest that ATP/ADP ratios may be good biomarkers of disease, especially since they correlate with CAG length. A particularly robust change identified by Strand in human HD muscle was in the levels of lactate dehydrogenase A. In one case, it was detectable even at a very early stage of disease.

To conduct more global searches for biomarkers, one participant suggested using metabolic profiling. David Lowe agreed and noted that, although the metabolome is only sparsely annotated, it has provided very promising results for some neurological disorders. He added that proteomic analyses are also worth pursuing.

Concluding remarks

As noted by David Housman, the HD 2004: Changes, Advances, and Good News (CAG)_n meeting was an important way-station on the path to a cure for HD. Unexpected findings broadened participants' view of HD and induced them to update their paradigms. For example, the "shortstop" mouse model alerted participants to the apparently critical importance of the exact sequence and length of a fragment for determining its toxicity, and indicated that neuronal inclusions are not necessarily pathogenic. *In silico* models of polyglutamine structure made participants consider alpha, in addition to beta, chains as possible configurations adopted by expanded polyglutamines. Although huntingtin's effects on transcription have long been appreciated, several new insights in this area also emerged. For instance, alterations in huntingtin's interactions with the basal transcription machinery were implicated in HD, and disruptions in the expression of genes involved in cholesterol synthesis were identified as contributors to pathogenesis.

The meeting also highlighted the importance of context in HD pathology. At the molecular level, the influence of the polyproline region on polyglutamine aggregation was noted. At the cellular level, participants discussed the roles of molecular crowding and molecular chaperones, as well as mutant huntingtin's ability to form aggregates in the nucleus and cytoplasm, but not in the ER nor in mitochondria. At the inter-cellular level, results were presented indicating that many HD-associated pathologies are observed in the cortices of mice expressing mutant huntingtin in all neurons of the brain, but not in mice expressing the protein only in the cortex. In addition, important roles for dopaminergic inputs and microglia were proposed.

The meeting also provided reasons for optimism at the clinical front. High levels of concordance between results obtained in animal models and humans were reported. In particular, the R6/2 mouse model mirrored human alterations in orexin levels, circadian rhythms, and mRNA expression patterns both in the brain and muscle. Advances in early intervention therapies, including the use of intrabodies and RNAi, were also presented, as well as important options for ameliorating HD symptoms, particularly tetrabenazine. Several participants predicted that combination therapies would be particularly effective, an expectation supported by data from studies using HDAC inhibitors in *Drosophila*. In addition, searches for biomarkers are underway and promising candidates are beginning to emerge.

In sum, the meeting revealed that the HD research community's efforts are paying off and the goal of developing a cure for HD is, slowly but surely, coming into sharper focus. Although eleven years ago no one could have predicted the biological complexities underlying the HD mutation, no one would have anticipated the remarkable progress in understanding huntingtin and HD either.