



HD 2006: CHANGES, ADVANCES, AND GOOD NEWS (CAG)_n

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Summary

Two years ago, the HD community was impressed with the magnitude and scope of the “HD 2004: Changes, Advances, and Good News (CAG)_n” meeting. Over 300 scientists, giving 196 presentations, attended the meeting—100 more than the previous meeting two years earlier. Remarkably, the number of attendees and presentations grew again in 2006: over 360 scientists participated giving 230 presentations. Equally impressive was the scientific progress achieved in two years, ranging from a greater depth of understanding of the mechanistic underpinnings of HD to new clinical advances.

A major lesson emerging from this year’s meeting was the importance of context. The focus of many studies in HD research has been understanding how the expanded polyglutamine region that characterizes mutant huntingtin triggers misfolding and toxicity. However, as underscored at this meeting, there are many non-polyglutamine determinants of toxicity. Participants presented data indicating that intramolecular, intermolecular, intracellular, timing and genetic contexts play key roles in shaping HD pathogenesis. Non-polyglutamine sequences within huntingtin, as well as the presence and conformational state of other cellular proteins are important. Also, huntingtin’s aggregation state is key for determining toxicity. Two years ago, the physical diversity of aggregates was discussed; this year, distinct physiological effects, including detrimental and beneficial ones, were correlated with the presence of specific aggregate forms. Furthermore, a major step in identifying genetic modifiers of HD was reported.

The extent of the contributions of cell autonomous mechanisms versus cell-cell interactions in HD pathology was another fundamental question addressed at the meeting. In 2004, a few observations suggested a potential role for cell-cell interactions. This year, new findings strongly bolstered this proposal, implicating alterations in synaptic transmission and corticostriatal trophic support. In addition, the likely importance of cell types that do not display visible signs of degeneration, particularly glial cells and interneurons, was underscored.

Participants also offered new insights into the transcriptional dysregulation associated with HD. For example, a link between transcriptional dysfunction and mitochondrial pathology was identified. In addition, studies of histone post-translational modifications in HD provided clues as to how histone deacetylase inhibitors, discussed as therapeutic candidates in 2004, might exert their effects. Other HD-associated pathologies examined at the meeting included bioenergetic alterations and the interaction of mutant huntingtin with cellular clearance systems.

In addition, participants expressed a renewed appreciation for comparisons of HD to other neurodegenerative disorders, particularly those with repeat expansions. For example, the potential implications of studies indicating that bidirectional transcription across repeats and RNA-mediated toxicity may contribute to pathology in myotonic dystrophy, spinocerebellar ataxia type 8, and Huntington’s disease-like 2 were discussed. Although the contribution of these processes to HD remains unknown, preliminary data suggest antisense transcripts are generated from the mutant huntingtin gene.

Important advances in therapeutic approaches presented in 2004 were also reported. For example, participants described advances in the optimization of delivery, stability and safety of RNAi molecules to reduce the expression of mutant huntingtin. Addressing a major question in the application of this approach, new findings indicated that silencing wildtype, in addition to mutant, huntingtin in adult organisms does not appear to have major deleterious effects. The feasibility of inducing the specific knockdown of mutant huntingtin was also discussed. At the protein level, participants described recent advances in the development of intrabodies, antibody fragments against huntingtin which can alter misfolding kinetics or interfere with mutant huntingtin’s access to other cellular components. Cystamine’s potential as a therapeutic candidate was re-visited, and potential advantages of its reduced form, cysteamine, were noted. Also, an update on the status of one of the small molecules identified in an anti-aggregation screen described in 2004, C2-8, was presented.

Participants also reported on several new candidate compounds and targets. For example, a compound that increases cellular ATP levels and one that inhibits chaperone function, show promise for decreasing mutant huntingtin toxicity. In addition, the anti-apoptotic factor XIAP was reported to confer neuroprotection and amelioration of motor symptoms in cell and animal models of HD. The sirtuins, a class of histone deacetylases that may help link metabolic rate to aging, were also discussed as therapeutic targets. Another promising new approach involves the stimulation of neurogenesis by adenoviral delivery of brain-derived neurotrophin factor (BDNF), together with Noggin, an inhibitor of the signaling pathway involved in astrocyte genesis. This treatment resulted in the reduction of motor symptoms and increased survival in a mouse model of HD.

Two therapeutic candidates in clinical trials, creatine and cell transplantation, were also discussed. Cell transplantation has yielded some positive results, but it is in the early stages of testing and several technical and theoretical challenges remain to be addressed. The creatine studies suggest that high doses of the compound have therapeutic effects, but larger studies are needed to confirm the findings.

Participants also discussed the identification of biomarkers of HD progression. Confirming predictions made in 2004 regarding the potential use of brain imaging techniques to monitor HD, this year, imaging of cortical thinning emerged as a powerful biomarker to streamline clinical drug trials. In addition, an update of the PRE-DICT-HD project suggests that cognitive tests may have the potential to reveal improvements in pre-symptomatic clinical trials. Advances in the potential use of neuropeptides as biomarkers were also presented. Moreover, 'omics' approaches yielded important new data. For example, a transcriptomics study suggested that gene expression patterns can be used to classify HD progression states. In addition, a metabolomics study revealed the importance of taking gender into account when searching for biomarkers of disease.

The development and use of new tools to answer key questions in HD was also described. For example, a system that automatically monitors the fates of cells over time and correlates the risk of death or dysfunction with other cellular parameters was presented. The system should help sort the myriad alterations observed in HD into causal relationships, compensatory changes, and epiphenomena. In addition, participants described new models of HD, including stem cell-based and conditional mouse models in which mutant huntingtin can be selectively turned on or off in specific cell types. Results illustrating the power of a biolistics-based slice model of HD to evaluate candidate drug targets were also presented. Moreover, participants were informed of the ongoing COHORT project which will generate a widely available source of biological samples from HD families linked to a longitudinal database including neurological data, as well as genetic, medical and family histories.

Mechanisms of pathogenesis

The importance of context

Many studies have examined how the expanded polyglutamine region that characterizes mutant huntingtin triggers misfolding and toxicity. However, there are many non-polyglutamine determinants of toxicity that contribute importantly to pathogenesis. Several participants presented data indicating that intramolecular, intermolecular, intracellular, timing and genetic contexts help shape mutant huntingtin's toxicity. Although issues of context were also addressed in the 2004 meeting, new insights and a greater depth of understanding were presented this year.

Intramolecular context

In 2004, Ron Wetzel described *in vitro* experiments showing that a stretch of prolines placed next to a polyglutamine region reduces aggregation. In the 2006 meeting, the effects of huntingtin polyproline regions were examined in greater depth and additional huntingtin domains and post-translational modifications were implicated in toxicity. Using yeast expressing a FLAG-tagged huntingtin construct, Susan Lindquist's team, for example, found that the proline-rich region adjacent to the polyglutamine stretch dramatically reduces mutant huntingtin toxicity, while the FLAG sequence unmask it. More generally, Lindquist noted that sequences flanking the polyglutamine region can convert a benign protein to a toxic species and vice versa.

Furthermore, Leslie Thompson observed that polyproline decreases the formation of visible aggregates *in vivo* and, in combination with polyglutamine, influences huntingtin's subcellular localization. Applying a systematic approach using GFP constructs of a huntingtin fragment including exon 1, Thompson and colleagues found that huntingtin's first 17 amino acids are required and sufficient for mitochondrial localization, but also favor association with the endoplasmic reticulum (ER) and Golgi apparatus. Association with the latter organelles is maximized in the presence of the polyproline region, which depends on the polyglutamine region to exert its effect.

A potential functional consequence of the activities of these domains, observed Thompson, is the disruption of calcium homeostasis in cells expressing mutant huntingtin. Although the chronic de-regulation of baseline calcium levels associated with HD does not seem to depend on huntingtin's first 17 amino acids, acute disruption of mitochondrial calcium levels in glutamate-challenged PC12 cells does. Indeed, isolated mitochondria incubated with mutant huntingtin fragments become uncoupled and depolarized. Thompson proposed that cytosolic targeting may be a normal function of huntingtin protein which becomes deleterious in the presence of expanded polyglutamine.

Moreover, current work from Ray Truant's group suggests that additional domains, as well as the phosphorylation status of huntingtin's N-terminus, also affect huntingtin's subcellular distribution and function. Using extensive analysis of point mutations and restoration deconvolution microscopy to monitor fluorescently labeled huntingtin constructs in living cells, Truant identified several sequences involved in huntingtin targeting. He characterized huntingtin's first 18 amino acids as a membrane targeting domain (hunMAD) which localizes to membranes of microautophagic vesicles, late endosomal vesicles and the endoplasmic reticulum in a manner that depends on ATP, temperature, and serine phosphorylation. Mutational analysis and circular dichroism spectroscopy indicate hunMAD is an amphipathic alpha-helix, similar to a targeting domain found in vesicle associated proteins known as VAMPs. In addition, Truant identified HEAT repeat sequences that allow huntingtin to enter the nucleus and, in previous work, reported the presence of a nuclear export signal in huntingtin's carboxy terminus. Taken together, Truant's data suggest that huntingtin may act as a shuttling scaffold protein, reversibly associating and dissociating from endosomes, perhaps including trafficking vesicles from distant synapses, and entering the nucleus where it can affect transcription. Truant's future study of hunMAD's interactions with other proteins should help examine this possibility.

Another illustration of the importance of intramolecular context was presented by Joan Steffan. Her data suggest that phosphorylation of two serine residues in huntingtin's N-terminus by the IκB

kinase complex (IKK) may enhance huntingtin degradation. At the 2004 meeting, Steffan reported that three lysines in huntingtin's N-terminus can be ubiquitinated or SUMOylated, and proposed these post-translational modifications may have important functional consequences. Now Steffan has established that the modifications can be regulated by IKK phosphorylation of serines 13 and 16 which are adjacent to the lysines. This phosphorylation results in decreased poly-ubiquitination, increased mono-ubiquitination and, conversely, increased poly-SUMOylation and decreased mono-SUMOylation. In addition, it may increase acetylation. Mutations that mimic these phosphorylations reduce huntingtin abundance and aggregation. Furthermore, they increase nuclear body localization in cell culture (albeit not in brain slices) and decrease toxicity in medium spiny neurons from acute striatal slices. Based on IKK's known activation by mutant huntingtin and its role in mediating the ubiquitination and degradation of FOXO3a, Steffan proposed IKK activation may represent an endogenous mechanism to fight HD. Consistent with this proposal, phorbol myristate acetate, an IKK activator, reduced huntingtin-mediated toxicity in striatal cells.

Although the fragmentation of mutant huntingtin and its implications for toxicity were only briefly addressed at the meeting, this facet of HD pathology has been extensively studied and discussed. Numerous reports have suggested that mutant huntingtin's toxicity arises from its cleavage and the accumulation of amino-terminal fragments within specific neuronal populations. Indeed, as noted by Al LaSpada, Michael Hayden's group recently reported that cleavage of mutant huntingtin at the caspase-6 site is required for neuronal dysfunction and degeneration. However, a complete understanding of this process is yet to emerge. For example, Michelle Gray noted that a recently created mouse model of HD which shares many similarities with human HD (see **Looking ahead: Tools that promise to advance HD research**), apparently shows no signs of fragment accumulation. At least under these circumstances, fragment accumulation seems unnecessary for the expression of HD pathology.

Intermolecular context

The most conspicuous intermolecular interaction in HD is the association of mutant huntingtin molecules to form intracellular aggregates. In 2004, Alexander Osmand described using biotinylated polyglutamine peptides to detect aggregation foci—sites that actively recruit monomeric polyglutamine molecules—in brain tissues from humans and various animal models of HD. He reported the widespread presence of AF, and suggested AF may be precursors to mature aggregates which ultimately reside in axons and dendrites. This year, Osmand described how the presence of AF and neuropil aggregates are related to disease progression. The new studies included cortical tissue from 7 presymptomatic HD gene carriers, enabling analysis of the early stages of disease. Osmand found a significant correlation between the product of qualitative scores for AF and aggregates, and the years to expected age of disease onset, based on age and repeat length. Extrapolation of the data suggests that the onset of histopathological changes may occur decades before the appearance of symptoms. Osmand also noted that cortical and striatal pathology were correlated with each other, as well as with CAG repeat length.

Dissecting the effects of aggregation on cells' health, however, has proved complicated. There are many varieties of aggregates that have different physiological effects. In 2004, Michael Hayden presented data from a fragment model of HD known as "shortstop" suggesting that the accumulation of large inclusions visible in the

light microscope are not always pathogenic. Data published shortly afterwards, and presented this year by Steve Finkbeiner, indicate that inclusions are, in fact, protective, leading to decreased levels of soluble forms of mutant huntingtin elsewhere in a neuron, and improved survival. Consistent with these findings, Jose Lucas reported that huntingtin inclusion bodies have no detectable effect on proteasome function, but filamentous aggregates inhibit it. Lucas's team showed that ubiquitinated filamentous huntingtin aggregates selectively inhibit the peptidase activity of the 26S proteasome in a non-competitive manner. The huntingtin filaments appear to interact directly with the 19S ubiquitin-interacting regulatory caps of the 26S proteasome. The findings help clarify conflicting results in which different researchers reported different effects of mutant huntingtin on proteasome function. Furthermore, Mathieu Lesort observed that amyloid-like aggregates, but not monomeric polyglutamine peptides, facilitate calcium-induced swelling and the disruption of mitochondrial function known as the mitochondrial permeability transition (MPT), of isolated liver mitochondria (see **Mitochondrial and bioenergetic alterations in HD**).

Mutant huntingtin's toxicity also depends importantly on the status of other cellular proteins. In 2004, Wetzel reported that aggregation of polyglutamine proteins increases when other macromolecules are present at high concentrations, a situation known as molecular crowding. Several additional inter-molecular effects were presented this year.

For example, the global status of the protein-folding quality control system was identified as playing an important role in defining mutant huntingtin toxicity. As described by Anat Ben-Zvi, a recent publication from Richard Morimoto's group showed that polyglutamine aggregation in *C. elegans* is enhanced by the presence of weak folding mutations in ostensibly unrelated proteins. Even a single destabilizing, temperature-sensitive mutation expressed at the permissive temperature, which is innocuous under normal conditions, can markedly increase polyglutamine aggregation. Conversely, polyglutamine expanded proteins can cause a loss of function of metastable proteins in a polyglutamine length-dependent manner. These effects are seen even with proteins that do not overlap in their subcellular locations. Thus, it appears that a variety of weak folding mutations are capable of affecting cellular folding homeostasis and modifying polyglutamine toxicity. Consistent with these observations, chaperones are important modifiers of huntingtin toxicity, and ER stress enhances toxicity in yeast and neurons.

In addition to these generalized effects, there is evidence for specific effects mediated by particular cellular proteins. For example, Lindquist noted that the presence and conformational state of other glutamine-rich proteins modulate huntingtin toxicity in a specific way. For example, the yeast prion protein Rnq1 contributes to huntingtin toxicity when it is in its prion state. In addition, amino acid sequences that modulate polyglutamine toxicity in *cis*, on the same huntingtin molecule, can also do so in *trans*, as part of other huntingtin molecules.

Profilin is another protein that seems to specifically affect mutant huntingtin toxicity. Profilin is a very abundant cellular protein most widely known for its actin sequestering activity and its function as a key integrator of signals leading to actin polymerization. As described by Kurt Fischbeck, profilin directly binds to the proline-rich tract adjacent to the polyglutamine stretch in huntingtin, and appears to suppress mutant huntingtin aggregation. Interestingly, profilin protein, but not mRNA, levels are reduced in cell and animal models of HD, as well as in HD postmortem brains. To assess whether correcting this reduction ameliorates HD toxicity, Fischbeck and colleagues overexpressed profilin in a *Drosophila*

model of HD. Improvements in eye function and morphology, and a modest, but significant, increase in lifespan were observed. The mechanism(s) by which profilin is mediating these effects remain(s) to be determined. As noted by Fischbeck, profilin has many ligands and is involved in many cellular functions, including actin dynamics, postsynaptic receptor turnover, vesicular trafficking, neurite outgrowth, RNA processing, and signal transduction.

Cellular context

Cellular context is also an important determinant of mutant huntingtin toxicity. Work presented by Veronique André and Roman Gonitel underscored this point. Using electrophysiological techniques to assess neuronal glutamate responses, André found that in R6/2 cortical cells, AMPA and NMDA peak currents are smaller than those of wildtype cells, whereas in R6/2 striatal cells, they are larger. Furthermore, in the cortex, magnesium sensitivity of NMDA receptors is increased at 40 days of age, whereas in the striatum, it is reduced at both 21 and 40 days. These differential changes indicate that HD affects specific neurons in unique ways.

Gonitel carried out a detailed characterization of somatic CAG instability—a process which results in changes in the number of CAG repeats, with a bias towards increased repeats. Their data indicate that patterns of CAG instability, which can be detected early in the disease process, differ significantly between various brain regions. Several mouse models, as well as humans, showed similar region-specific signatures. Of particular interest, the striatum is one of the brain regions with the highest rate of CAG expansion and expanded alleles are greatly favored for transcription. In addition, whereas all neuronal cells examined in 6-month-old R6/1 mice revealed instability, non-neuronal cells had both stable and unstable components. Although somatic CAG instability is not required for disease expression—mouse models of HD with no instability still display pathology and behavioral phenotypes—Gonitel noted that instability may accelerate disease progression and could contribute to the differential vulnerability of various cell types.

Genetic context

At the 2004 CAG meeting, participants predicted genetic modifier studies would significantly contribute to understanding HD and identifying new therapeutic targets. Several approaches to search for these modifiers, in a variety of systems including yeast, *C. elegans*, *Drosophila*, knock-in mice, and humans, were described in the poster sessions. Now, at the 2006 meeting, results from a human study were presented. As explained by Michael Andresen, CAG repeat length accounts for approximately 70% of HD's variability in age of onset, including individuals of all CAG repeat lengths. It only accounts for 44% of the variability, however, in individuals with moderate (40–50) repeat lengths. The residual variance appears to be a heritable trait, suggesting the existence of genetic modifiers of HD age of onset. Based on their analyses, Michael Andresen, Javier Gayan, Denise Brocklebank, Stacey Cherny, Lon Cardon, David Housman and Nancy Wexler undertook a genome-wide single nucleotide polymorphism (SNP) linkage analysis in large Venezuelan HD kindreds. Working with over 800 samples and close to 6000 SNP markers, the researchers had to separate the large, interconnected family pedigrees into subpedigrees to facilitate computer analyses. Loci with LOD scores greater than two (odds greater than 100 to 1 in favor of genetic linkage) were identified on chromosomes 2, 4, 5, 6 and 12. The loci on chromosomes 2 and 6 appear particularly promising because of their high LOD

scores (odds greater than 4, or over 10,000 to 1 in favor of linkage, on chromosome 2). The data replicate previous findings and reveal novel loci. To extend these findings, the team plans to conduct association analyses of candidate genes.

Timing context

Additional factors determining polyglutamine toxicity are aging and disease progression. Many cellular functions change with aging and this may affect HD pathology. For example, cellular folding capacity appears to decline with age as observed by Anat Ben-Zvi who reported age-dependent misfolding of temperature-sensitive mutants. Moreover, lifespan regulators, including age-1 and daf-16, are known to regulate protein homeostasis, as well as polyglutamine aggregation. And, as noted by Kimberly Kegel, aging is associated with many transcriptional changes, some of which could have important effects on HD pathology. Len Guarente proposed that targeting proteins that slow aging, particularly the sirtuin Sir2 which helps link metabolic rate and aging, may have therapeutic value (see **Targeting pathways that regulate aging and metabolism**).

It is also important to note that HD-associated alterations change during disease progression. Data presented by Veronique André from Michael Levine's group, for example, highlighted the evolving status of synaptic function. Monitoring glutamate receptor responses in dissociated pyramidal cortical neurons and striatal medium spiny neurons from R6/2 mice at different stages of disease, she and her colleagues found that NMDA and AMPA receptor responses change over time. Both responses, in both cell types, were altered very early in the disease process (21 and 40 days—before symptoms are evident), but normalized later (80 days).

A striking illustration of how different phases of a disease may involve the action of mutant proteins in different cell types was provided by Don Cleveland's work on amyotrophic lateral sclerosis (ALS). Using mice carrying a conditional mutation of the superoxide dismutase gene which causes ALS, Cleveland's team found that expression of the gene in motor neurons was a primary determinant of disease onset and of an early phase of disease progression. However, later disease progression was dramatically affected by expression of the mutant enzyme in microglia. Noting that previous attempts to treat ALS with gene therapy probably failed because of incorrect cell targeting, Cleveland said his group is now testing the therapeutic potential of pumping DNA antisense oligonucleotides against superoxide dismutase into the cerebrospinal fluid (CSF) where they can reach both neurons and glia (see **Searching for therapeutic targets and compounds**).

Cell-autonomous pathology versus cell-cell interactions

Another major question in the study of HD is whether mutant huntingtin mediates its toxic effects through cell autonomous mechanisms, cell-cell interactions, or both. For example, André's results may be explained by mutant huntingtin having direct effects on glutamate responses in both cortex and striatum independently, or a cortical alteration in glutamate receptor function may cause decreased spontaneous activity in the striatum which, in turn, may result in a compensatory striatal response involving increased glutamate receptor function. It is also possible that both of these processes contribute to pathology.

To help address this issue, in 2004, William Yang described the generation of Cre/LoxP conditional mouse models of HD that express mutant huntingtin in either all neurons in the brain

(pan-neuronal model), or only in the vulnerable cortical projection neurons (cortical model). This year, Yang presented additional models, including one in which mutant huntingtin expression is activated exclusively in striatal projection neurons (striatal model). The data indicate that progressive motor deficits and robust neuropathology occur in the pan-neuronal model, but not in the cortical or striatal models. Surprisingly, in these models, only mild cell-autonomous pathologies, including aggregation, are observed. Based on his observations, Yang proposed a two-hit model in which both cell autonomous processes, as well as cell-cell interactions contribute to HD pathology.

To further investigate the roles of specific cell types and cell-cell interactions, Yang's team has developed a transgenic model (BACHD) in which mutant huntingtin can be selectively switched off in specific cell types. As described by Michelle Gray, the transgene is a bacterial artificial chromosome (BAC) that contains a full-length copy of the human huntingtin locus with a 103 mixed CAG/CAA repeat. To control its expression, mutant huntingtin exon 1 is flanked by two *Lox P* sites. The researchers have generated several mouse lines and shown that the transgene is expressed in all neurons and can rescue embryonic lethality of *Hdh* knockout mice. Several key features of human HD are recapitulated in these animals (see **Looking ahead: Tools that promise to advance HD research**), and pathological cell-cell interactions are evident. Consistent with Michael Levine and colleagues' findings, cortical and striatal cells exhibit electrophysiological alterations suggesting dysfunction of the cortico-striatal pathway. Of particular interest, aberrant cortical inhibition appears to occur very early in the disease, suggesting early cortical interneuron dysfunction.

To extend these findings, Yang and colleagues are turning off mutant huntingtin in specific cell populations by crossing BACHD mice with mice carrying the *Cre* gene under the control of various promoters, including specific promoters for cortical pyramidal cells, striatal medium spiny cells, cortical parvalbumin interneurons, astrocytes and microglia. In addition, Yang is working with Nat Heintz's GENSAT mice—transgenic BAC mice in which endogenous coding sequences have been replaced by the eGFP reporter gene—and setting up cell type-specific microarray analyses using fluorescence activated cell sorting (FACS) of brain tissue from mice with labeled striatal cells.

Another approach to examine the contributions of cell autonomous effects and cell-cell interactions in HD pathology was presented by Michelle Ehrlich. Her team created transgenic mice which express a fragment or full-length copy of mutant huntingtin predominantly in the medium spiny neurons of the striatum using "striatal-specific" fragments of the *DARPP-32* gene to direct transgene expression. Her preliminary data, based on two transgenic animals, suggest that the formation of nuclear inclusions and early gliosis in the ventral striatum are cell autonomous processes. In addition to increasing the sample size and continuing ongoing studies of behavioral traits, Ehrlich plans to generate additional transgenic mice to control for compensatory changes that may occur during development.

Altered cell-cell interactions: Synaptic transmission

Several participants suggested disruptions in synaptic transmission may be key to HD pathology. As previously noted (see **Cellular context**), Veronique André and colleagues observed HD-associated alterations in glutamate responses in cortex and striatum. Consistent with these findings, Lynn Raymond's team observed increased NMDA receptor-mediated calcium entry and apoptosis

in striatal medium spiny cells from HD mice carrying mutant huntingtin constructs with 72 CAG repeats (YAC72) or 46 repeats (YAC46). However, an examination of cells derived from mice carrying constructs with 128 repeats (YAC128) yielded conflicting results. Like the medium spiny cells of YAC46 and YAC72 mice, those of YAC128 mice have enhanced levels of NMDA receptor-mediated apoptosis and disturbed calcium dynamics. However, the electrophysiological responses of NMDA receptors in YAC128 striatal cells appear normal, with peak currents very similar to those of wildtype cells.

To resolve this apparent paradox, Herman Fernandes and colleagues used whole-cell patch recordings and fluorescent imaging to monitor calcium dynamics and mitochondrial potential in YAC128 cells. The data indicate that the mitochondria of YAC128 medium spiny neurons are impaired in their capacity to handle NMDA receptor-mediated calcium entry. Correspondingly, these cells are more sensitive than wildtype cells to blockers of the mitochondrial permeability transition (MPT) which reduce peak calcium levels downstream of NMDA receptors. Fernandes proposed that these early alterations in calcium handling may be relevant to HD associated neuronal dysfunction.

The striatum's dense dopaminergic innervation may also contribute to HD pathology. In 2004, Jocelyne Caboche reported that low doses of dopamine can act synergistically with mutant huntingtin to induce apoptosis and induce aggregate formation in striatal cells expressing mutant exon 1. This year, Tie-Shan Tang reported that dopamine potentiates glutamate-induced apoptosis in YAC128 medium spiny neurons but, in contrast to Caboche's findings, dopamine alone does not enhance apoptosis. In addition, whereas Caboche's study implicated D2 dopamine receptors, Tang's implicates D1 receptors. Consistent with the involvement of D1 receptors, Tang noted that the activation of mGluR1/5 and NMDA glutamate receptors is required for dopamine's apoptotic effects. Differences in model systems may explain these apparently conflicting results. Indeed, as noted above, a difference in CAG repeat length can result in important pathogenic differences.

To identify the molecular mechanisms by which mutant huntingtin affects synaptic transmission, Eliana Romero and colleagues are characterizing genetic suppressors of synaptic transmission in a *Drosophila* model of HD. In this model, which expresses full-length human huntingtin with 128 CAG repeats, the efficiency of synaptic vesicle release, as well as intracellular calcium levels, are increased. Romero found that partial loss-of-function mutations in specific neurotransmitter secretion proteins helped normalize transmitter release. In addition, similar mutations in voltage-gated calcium channels suppress both the intracellular calcium and neurotransmitter release phenotypes.

Other searches for determinants of HD pathology that have not focused *a priori* on synaptic transmission have also identified synaptic proteins as potential mediators of huntingtin toxicity. For example, Robert Hughes conducted high throughput screens for huntingtin-associated proteins and identified 234 novel interactors. Forty-eight of these had *Drosophila* orthologs which act as modifiers of neurodegeneration in a fly model of HD and, of these, 12 had both enhancer and suppressor alleles. Many of the proteins in this highly selected set of HD modifiers are involved in synaptic transmission, including SNAP proteins, syntaxin 1A, t-SNARE proteins, and a synaptic voltage-gated calcium channel. Of particular interest, a loss-of-function mutant of syntaxin 1A suppressed eye abnormalities and motor alterations, and enhanced fly survival. Moreover, Hughes noted that RNA silencing of syntaxin 1A in mammalian brain slices reduced exon-1 *Htt*-associated neurodegeneration.

Another study that revealed a possible involvement of synaptic proteins in HD pathology was presented by Kevin Jones. His group compared mice lacking cortical brain-derived neurotrophic factor (BDNF) to HD mice, reasoning they may share informative similarities since the striatum's supply of cortical BDNF is reduced in HD, as described by Elena Cattaneo and colleagues. The BDNF mutant mice displayed a progressive clasping phenotype characteristic of some HD models, reduced striatal volume, and striatal cell loss (35%) in animals over a year old. In addition, Jones reported a dramatic reduction in dendritic spine density 35 days after birth, suggesting an early and significant loss of striatal synapses. When the researchers used a gene ontology analysis to compare patterns of gene expression in these BDNF-depleted mice to HD mice, several synaptic genes were identified, including calcium signaling genes and neuroactive ligand receptors.

Structural synaptic proteins were also implicated in HD pathology. Ihn Sik Seong from Marcy McDonald's group reported that the cell-adhesion molecule N-cadherin, which is involved in synaptic function and neuronal cell survival, decreases with age in knock-in HD mice (HdhQ111/111), starting at approximately 12 months. Seong found that N-cadherin in mutant striatal cells is degraded more rapidly in response to ATP depletion than in normal striatal cells. This is particularly relevant to HD in light of recent observations that cellular ATP and ATP/ADP ratios are reduced early in the presymptomatic disease process. Consistent with these findings, mutant striatal cells show an impaired capacity for self-adhesion which is dependent, as is N-cadherin, on calcium.

Altered cell-cell interactions: Trophic support

One cell-cell interaction that several studies have implicated in HD pathology is the cortical delivery of BDNF to the striatum. At the 2004 meeting, Elena Cattaneo noted that striatal BDNF is produced in the cortex where its transcription is stimulated by wildtype huntingtin's sequestration of the repressor element-1 transcription factor (REST) which inhibits the Neuronal Restrictive Silencer Element (NRSE). This regulatory activity is compromised in cells expressing mutant huntingtin, such that the expression of BDNF and other neuronal genes is downregulated (see ***Transcriptional abnormalities associated with HD***). In addition, Frederic Saudou reported in 2004 that huntingtin specifically enhances the transport of vesicles containing BDNF along microtubules. Expressing mutant huntingtin dampened BDNF transport and resulted in the loss of neurotrophic support and neuronal toxicity.

Testing the physiological implications of these results, Jones and colleagues reported at this meeting, that mice lacking BDNF expression in the cortex resemble HD mice in several important ways (see ***Altered cell-cell interactions: Synaptic transmission***). Although mutant huntingtin has other effects besides reducing BDNF, Jones noted that identifying changes that are common to HD and cortical BDNF-deprived mice might reveal valuable therapeutic targets. Jones is now planning to deliver exogenous BDNF to the cortex of his knockout mouse to assess whether the observed phenotypes are reversible.

A global comparison of gene expression in humans and animal models of HD strengthened the hypothesis that BDNF alterations are important in HD. Andrew Strand discovered that the striatal expression pattern of Jones's BDNF knockout mice is highly concordant with that of human HD caudate nucleus. The expression patterns of R6/2 mice and mice treated with 3-nitropropionic acid—a toxin that induces striatal damage similar to HD—also showed significant positive correlation with human HD mRNA

profiles, but the BDNF knockout profile yielded the best concordance as assessed by three different statistical analyses. Comparing human and R6/2 profiles, similarity was most apparent in gene activities that are decreased in HD, whereas comparing 3-NP and human profiles, similarity was greatest in gene activities that are increased in HD. The BDNF knockout profile was equivalent to the R6/2 profile in mirroring gene profiles decreased in HD, and to the 3-NP profile in mirroring gene profiles increased in HD.

Altered cell-cell interactions: Glia

Participants also noted that non-neural cells may play important roles in HD pathology through cell-cell interactions. In 2004, Paolo Guidetti reported that the excitotoxin quinolinic acid and its bioprecursor, 3-hydroxykynurenine (3-HK), both produced by microglia, are elevated in HD mice brains and in the neostriatum and neocortex of HD patients at early stages of disease. Carrying out a screen for mutant huntingtin suppressors in yeast, Paul Muchowski and colleagues now report that the majority of the suppressors they've identified converge on the kynurenine pathway. Although they act on different upstream targets, they result in downregulation of quinolinic acid and 3-HK. In addition, microarray analyses revealed transcriptional dysregulation of the kynurenine pathway in HD mice. As noted by Muchowski, in humans, only microglia express a key enzyme in the kynurenine pathway. Furthermore, several studies have described reactive microglia in HD brains, as well as alterations in their morphology and numbers. The histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA), a transcriptional regulator which has been shown to be neuroprotective in several HD models, blocked activation of the kynurenine pathway in isolated microglia and in HD mice. Also, the kynurenine 3-hydroxylase inhibitor Ro61-8046 had beneficial effects in HD mice.

As noted by LaSpada, work from Xiao-Jiang Li's lab has also implicated glial cells in huntingtin toxicity. Using a neuron-glia coculture system, Li and colleagues observed that wild-type glial cells protected neurons against mutant huntingtin-mediated neurotoxicity, whereas glial cells expressing mutant huntingtin increased neuronal vulnerability. The researchers proposed that mutant huntingtin expressed in glial cells results in decreased glutamate uptake which contributes to neuronal excitotoxicity.

Interestingly, the involvement of glia in neuronal pathology has also been reported in other neurodegenerative disorders. As previously described, Don Cleveland identified microglia as key mediators of ALS progression, and LaSpada noted a potentially major role for Bergmann glia in spinocerebellar atrophy type 7 (SCA7).

HD as a transcriptionopathy

Transcriptional abnormalities associated with HD

As noted by Al LaSpada, most polyglutamine disorders, including HD, can be considered transcriptopathies. In 2004, Lesley Jones and Jim Olson's group reported results from a global assessment of gene expression in HD, which included various human HD brain tissues, and a few mouse models. The results indicated that the caudate had the highest number and greatest magnitude of transcriptional changes, with major disruptions in the expression of proteins involved in neurotransmission and intracellular signaling.

Extending these studies, this year, Ruth Luthi-Carter described a systematic comparison of striatal mRNA profiles from post-mortem HD human caudate to seven animal models of HD, which included animals carrying N-terminal fragments of human mutant

huntingtin and knock-in animals expressing mutant mouse huntingtin. Knock-in animals had smaller magnitude changes than transgenic animals, but there were no discernable qualitative differences between the two types of models. The best concordance of orthologous gene expression patterns and the best permutation test outcomes were obtained from 14-week-old R6/1 mice, 6-week-old R6/2 mice, 22-month-old CHL2 mice, and 18-month-old Hdh92Q mice. Changes that were concordant across multiple models closely mirrored changes in human caudate. Taken together with Andy Strand's data (see *Altered cell-cell interactions: BDNF*), Luthi-Carter's results indicate that transcriptional changes observed in a wide variety of animal models are similar to those associated with human HD.

Gene expression datasets as those described above are also shedding light on disease mechanisms. Confirming predictions made in 2004, bioinformatics analyses of these data are yielding important insights into HD. For example, as previously described, Strand's analyses provided new support for the hypothesis that a lack of trophic support is an important contributor to HD pathology.

Moreover, using a bioinformatics algorithm known as Gene Set Enrichment Analysis (GSEA) to analyze several expression datasets, Dmitri Krainc and colleagues identified a new link between transcriptional dysregulation and mitochondrial pathology in HD. Krainc's team found that the expression pattern of caudate tissue from HD brains significantly overlaps with that of mice lacking PGC-1 α , a transcriptional co-activator which acts as a master regulator of several metabolic processes, including mitochondrial biogenesis and respiration. PGC-1 α knockout mice develop neuropathological abnormalities that primarily involve the striatum. Moreover, Krainc observed that PGC-1 α levels are decreased 6-fold in medium spiny neurons isolated from HD mice, whereas PGC-1 α expression is increased 47-fold in HD interneurons. Krainc suggested that decreased PGC-1 α may contribute to medium spiny cell vulnerability, whereas increased PGC-1 α may help protect striatal interneurons. Consistent with these observations, crossing PGC-1 α knockout mice with HD knock-in mice enhances HD pathology and, conversely, inducing expression of PGC-1 α in HD cultured cells and *in vivo* partially reverses HD toxicity. Krainc concluded that, in addition to exerting direct effects on mitochondria (see *Mitochondrial and bioenergetic alterations in HD*), mutant huntingtin interferes with medium spiny cells' capacity to respond to increased energy demands by inhibiting PGC-1 α up-regulation.

Candidate mechanisms by which mutant huntingtin disrupts transcription

Participants also addressed the potential mechanisms by which mutant huntingtin disrupts transcription. In 2004, mutant huntingtin's anomalous interactions with several transcriptional proteins, including components of the basal transcription machinery TAFIIS and TFIIF, the transcriptional regulator CBP, and transcription factors Sp1 and REST were discussed.

In 2006, an update on mutant huntingtin's interaction with REST was presented. As previously mentioned, mutant huntingtin is compromised in its ability to sequester REST, a transcriptional repressor which binds to the neuronal silencer NRSE. In 2004, Elena Cattaneo described using chromosome immunoprecipitation (ChIP) assays to monitor REST binding to BDNF and synapsin 1 NRSEs. She found that REST binding is increased in neurons expressing mutant huntingtin as compared to controls. This year, Chiara

Zuccato reported similar results for other NSRE-containing genes (such as *chrn4* and *Drd3*), as well as corresponding reductions in their mRNA levels which could be rescued with transient transfections of a REST dominant-negative mutant. The negative transcriptional effect of mutant huntingtin appears to be due to a loss of wildtype huntingtin function since models with reduced wildtype huntingtin—embryonic stem cells and a cortical conditional mutant—yielded similar results. To systematically assess the effects of mutant huntingtin on many NRSE-containing genes at once, Zuccato has set up parallel NSRE screens including 500 genes, in effect, conducting ChIP on chips. So far, she has observed approximately 200 genes with increased REST occupancy in HD cortex.

Alterations in the regulation of chromatin structure by histones may also contribute to HD transcriptional pathology. In 2004, participants described histone deacetylation (HDAC) inhibitors as therapeutic candidates for HD. Histone acetylation helps make tightly packed chromatin accessible to transcription factors, thereby increasing gene transcription. However, exactly how HDAC inhibitors benefit HD has remained unclear—it is even uncertain whether the inhibitors' effects on HD involve histones or are instead due to changes in the acetylation of other proteins. This year, Jang-Ho Cha and colleagues provided data on the post-translational status of histones in HD, including insights into histone acetylation and how HDAC inhibitors may ameliorate HD pathology. Using ChIP analysis combined with real-time PCR, the researchers found hypoacetylation of histone H3 proteins associated with downregulated genes in two HD striatal cell lines. Treatment of the cells with an HDAC inhibitor increased the association of acetylated histones with downregulated genes and improved mRNA abnormalities.

Insight into the HDAC types relevant to HD was provided by Linda Kaltenbach who is testing the effects of individually silencing and overexpressing 16 of the 18 rodent HDAC genes. In 2004, Larry Marsh reported that reducing HDAC classes I and III, but not II, in *Drosophila* alleviated HD toxicity. However, the roles of mammalian HDACs in HD remained unknown. Kaltenbach's work addresses this question by using a striatal brain slice model of HD developed by Don Lo and colleagues which allows researchers to perform co-transfections at very high efficiencies using biolistics (see *Looking ahead: Tools that promise to advance HD research*). Kaltenbach has used shRNAs to silence HDAC genes and transfections of CMV-driven cDNAs to overexpress the genes. Although the work is still in progress, Kaltenbach noted that shRNAs against HDAC4 and 10 increased medium spiny neuron health, and cDNAs coding for HDAC7 and 3 enhanced cell death. Surprisingly, a cDNA coding for HDAC4 suppressed cell death.

In addition to acetylation, histone function can be regulated by ubiquitination and new data from Jang-Ho Cha's group suggests this post-translational modification may also play a role in HD pathology. Noting that monoubiquitylation of histone H2A is involved in gene silencing, while monoubiquitylation of histone H2B results in transcriptional activation, Cha reported that the levels of ubiquitinated H2A are increased in R6/2 brains compared to controls, while those of ubiquitinated H2B are decreased. In addition, the promoters of the downregulated genes in R6/2 mice have increased association with ubiquitinated H2A, but decreased association with ubiquitinated H2B. In conditional models of HD, induction of mutant huntingtin expression induced increased association of ubiquitinated H2A with HD downregulated genes. In addition, when ubiquitinated H2A was reduced in HD cells by knocking down Ring2, a protein required for H2A ubiquitination, the mRNA levels of downregulated genes were partially corrected.

Mitochondrial and bioenergetic alterations in HD

Many studies have implicated mitochondrial disruptions and alterations in energy metabolism and oxidative stress in HD pathology, including energy metabolic disruptions in the striatum, decreased ATP levels, and impairments in mitochondrial complex II and III function, aconitase activity, and glucose metabolism. Also, lactate levels are increased in the basal ganglia and parts of the cortex, and, in muscle, phosphocreatine levels are decreased.

A new insight presented at the meeting that helps integrate several of these findings is the discovery presented by Dmitri Krainc of the HD-associated downregulation of a master regulator of metabolism and mitochondrial function, PGC-1 α (see *Transcriptional abnormalities associated with HD*). LaSpada noted that his group's work—indicating a progressive decline in body temperature and an inability to thermoregulate properly in various mouse models of HD—has also led them to study PGC-1 α 's role in HD.

In addition, several studies have reported direct effects of mutant huntingtin on mitochondria. In 2004, Mathieu Lesort described huntingtin localizing to the outer mitochondrial membrane and alterations in calcium handling in isolated mitochondria incubated with mutant huntingtin fragments. This year, as previously mentioned, Lesort reported on new studies to determine the toxic effects of specific conformations of polyglutamine peptides on mitochondria. Monitoring the swelling of isolated liver mitochondria by measuring changes in turbidity, Lesort found that an exon 1 fragment of huntingtin with 65 glutamines facilitates calcium-induced swelling. The effect can be prevented by cyclosporin A, an inhibitor of the mitochondrial permeability transition, and replicated by amyloid-like aggregates, but not monomeric polyglutamine peptides.

In an effort to identify mitochondrial protein(s) that interact with mutant huntingtin, Pier Mastroberardino described using affinity chromatography to pull down proteins from isolated mitochondria incubated with polyglutamine peptides. Predicting that the level of polyglutamine binding to mitochondria is low and the interaction weak, Mastroberardino used mild solubilization methods and subsequently characterized eluates using two-dimensional electrophoresis and mass spectrometry. The experiments identified aspartate aminotransferase—an enzyme involved in intermediary energy metabolism and in regulation of the transmitter pool of glutamate—as a polyglutamine-binding protein. Moreover, Mastroberardino observed that the enzyme's activity, although not its protein level, was reduced in the brains of HD knock-in mice.

Another approach to investigate HD-associated disruptions in energy metabolism and oxidative stress was presented by Elisa Fossale. In 2004, Marcy McDonald reported that HD cortex and striatum have decreased ATP/ADP ratios at early stages of disease, and the ratios correlate well with CAG length in human lymphoblastoid cells. Moreover, both ADP and ATP levels are low in HD mitochondria. To extend these findings, Fossale and colleagues characterized small molecules that alter metabolism in yeast. Of 96 compounds, three were found to increase, while 5 decreased, ATP levels in mutant striatal cells. One of the molecules that increased ATP was tricylcodecan-9-yl xanthate (D609)—a phospholipase inhibitor, but also a potent antioxidant that mimics glutathione. D609, as well as glutathione, completely protected mutant striatal cells against EOP-23E, a small molecule that decreases ATP levels. Deleting or inactivating D609's thiol group, which is critical for the molecule's antioxidant activity, abrogated its protective effects. Taken together, the results implicate oxidative stress early in HD pathogenesis.

Clearance and mutant huntingtin: A two-way street

Huntingtin is a substrate for cellular clearance systems but, in its mutant form, it can also be a disruptor of protein degradation. Both aspects of this relationship were discussed at the meeting, as well as their therapeutic implications.

In 2004, Prasanna Venkatraman presented data on the clearance of huntingtin, noting that although eukaryotic proteasomes can cleave single glutamine-glutamine bonds, they are unable to cut polyglutamine stretches. Thus, when degrading polyglutamine proteins, eukaryotic proteasomes release undigested polyglutamine peptides. This year, Alfred Goldberg provided the missing piece to this puzzle describing enzymes capable of degrading polyglutamine fragments. Although most peptidases known to degrade typical proteasome products are unable to digest polyglutamine sequences, the puromycin-sensitive aminopeptidase (PSA), which is abundant in brain, is capable of doing so. Silencing the PSA gene or using puromycin to block its activity inhibits polyglutamine degradation in cell extracts. Furthermore, PSA can degrade polyglutamine fragments that are up to 40 glutamines long. However, the process is inefficient. Searching for alternative degradation paths, and because autophagy has been implicated in HD, Goldberg and colleagues also examined lysosomal enzymes. Using inhibitors and recombinant enzymes, they identified the cysteine proteases cathepsins L and X as critical for polyglutamine degradation.

Regulators of huntingtin degradation, which may provide new therapeutic targets, are also beginning to emerge. As previously noted, Joan Steffan reported that phosphorylation of serines 13 and 16 in huntingtin's N-terminus by the I_B kinase complex (IKK) may enhance huntingtin degradation (see *Intramolecular context*).

Participants also presented findings indicating mutant huntingtin interferes with cells' clearance systems. As previously noted, Jose Lucas's group reported that huntingtin filaments, although not inclusion bodies, interact directly with the 19S ubiquitin-interacting regulatory caps of the 26S proteasome to inhibit proteasome activity. In addition, Susan Lindquist noted that a mutant huntingtin fragment expressed in yeast blocks ubiquitin fusion degradation, as well as endoplasmic reticulum associated degradation (ERAD). Consequently, the unfolded protein response is activated and cells become sensitized to ER stress. Consistent with these findings, overexpression of ERAD proteins ameliorates mutant huntingtin toxicity.

Loss-of-function alterations

Many of the pathological mechanisms described above involve gain-of-function effects. However, loss-of-function effects also appear to contribute to HD pathology. For example, previous studies by Scott Zeitlin and colleagues indicate that inactivating wild-type huntingtin expression in mouse forebrain results in progressive neuronal degeneration. In addition, Sheng Zhang observed weak, but reproducible, phenotypic alterations in *Drosophila* null mutants with deletions of huntingtin genomic DNA. Although the mutant flies were viable, fertile, and without obvious morphological defects—including no abnormalities in adult eye structure or eye development—they had decreased locomotor activity compared to controls as they aged, and decreased lifespans. To understand the defects underlying these alterations and gain new insights into huntingtin's wildtype function, Zhang has begun examining the morphologies of neuromuscular junctions, muscle patterning and the nervous system. Although he has observed no gross morphological defects, he found reduced axonal complexity.

HD may affect wildtype huntingtin function in various ways. As previously mentioned, Cattaneo's findings show that wildtype, but not mutant, huntingtin sequesters the repressive transcription factor REST/NSRF that regulates the expression of a wide variety of neuronal genes, including BDNF. Thus, the reduced level of wildtype protein in HD brains can result in major transcriptional alterations. In addition, mutant huntingtin may affect normal huntingtin function by sequestering the wildtype protein, inducing its proteolysis, or forming dysfunctional complexes with other proteins that normally interact with wildtype huntingtin.

In 2004, Zeitlin presented his team's efforts to address this issue by generating mice carrying FLAG-tagged huntingtin alleles that allow discrimination between normal and mutant huntingtin. Initial results derived from these mice were presented this year. As revealed by pull-down assays and co-immunoprecipitations using brain extracts, huntingtin constructs with only 7 glutamines interact very little with mutant constructs bearing 140 glutamines, and the interactions are detectable only until the mice reach 24 months of age. However, huntingtin constructs with 20 glutamines show clear interactions with those carrying 140 glutamines at 4, 6 and 12 months of age. Using a filter-trap assay to examine insoluble aggregates, Zeitlin found small amounts of wildtype huntingtin trapped in these fractions. The team plans to extend these studies and examine the subcellular localization of the different huntingtin constructs. Preliminary data suggest this will be complicated, however. Among other things, the accessibility of the FLAG epitopes varies with their location—they appear to be masked or clipped off in the nucleus, but not in the neuropil.

Commonalities with other repeat diseases

Many of the pathological mechanisms described above also appear to play roles in other degenerative diseases. As noted by David Housman, shortly after the realization that repeat expansions were associated with several degenerative disorders, many researchers turned their attention to these related diseases in search of clues to HD pathology. However, interest waned after a few years, perhaps because of an increasing preoccupation with the vulnerability of striatal medium spiny cells in HD. Now the pendulum may be swinging back, as growing numbers of cellular and molecular similarities emerge, suggesting that much can be learned from comparative studies.

Polyglutamine disorders

Illustrating the fundamental kinship between polyglutamine diseases, Al LaSpada noted it is possible to recapitulate several features of these disorders by introducing a long polyglutamine stretch into hypoxanthine phosphoribosyl transferase, a protein which does not normally have a polyglutamine domain. Mice expressing this protein develop an adult-onset neurologic phenotype that includes lack of coordination, involuntary limb claspings, seizures, and premature death. In addition, they have widespread ubiquitinated neuronal intranuclear inclusions.

LaSpada added that polyglutamine disease has fundamentally two faces: selective vulnerability (as occurs in adult onset HD) and non-selective degeneration (as occurs in juvenile HD). Another hallmark of polyglutamine disease is its multi-valency: polyglutamine expansion is toxic to various cell processes, and includes both gain- and loss-of-function effects.

A more detailed level of comparison also reveals similarities in the pathways affected by polyglutamine toxicity. For example, proteolytic cleavage of mutant proteins seems to play a role in several of the polyglutamine disorders. As previously mentioned, mutant huntingtin fragmentation has been repeatedly implicated in HD pathology. In addition, LaSpada noted that his team, in collaboration with Lisa Ellerby, recently characterized a 55kDa fragment of ataxin-7 that localizes to the nucleus and forms aggregates. Transgenic mice with a mutation in one of the cleavage sites responsible for generating this fragment exhibit improved neurological phenotypes and increased lifespan.

As in HD, protein and cellular contexts are critical determinants of toxicity in other polyglutamine disorders. For example, a serine phosphorylation site in ataxin-1 appears to contribute importantly to inclusion formation and pathogenesis. The involvement of cell-cell interactions also seems to be a recurring theme. LaSpada noted, for example, the recent discovery of a key role for Bergmann glia in spinocerebellar atrophy type 7 (SCA7). Apparently, mutant ataxin-7 impairs the function of a glutamate transporter in these cells which, in turn, causes neurodegeneration. The findings are reminiscent of the observations by Xiao-Jiang Li and colleagues indicating that glial cells expressing mutant huntingtin increase neuronal vulnerability.

In addition, LaSpada noted that most polyglutamine disorders can be considered transcriptopathies. Indeed, most polyglutamine proteins are either transcription factors or co-regulators, or proteins capable of interacting with these proteins. Several pathological transcriptional interactions have been described in HD, involving, for example, transcription factors REST/NSRF, Creb-binding protein, and Sp1, as well as components of the basal transcription machinery, TAF_{II}5 and TFIIF. Similarly, transcriptional interference has been identified as a key mechanism underlying SCA pathology.

Abnormal mitochondrial function and energy production, as well as loss-of-function alterations, are additional features shared by polyglutamine disorders. Bioenergetic disruptions have been described in the SCA disorders and, as previously noted, mutant huntingtin affects the transcription of genes involved in metabolism and mitochondrial function, in addition to directly interacting with mitochondrial components. As suggested by Cattaneo's, Zeitlin's and Zhang's data, loss-of-function disruptions are also important contributors to HD. Similar findings have emerged for other polyglutamine disorders, including spinal and bulbar muscular atrophy (SBMA) and SCA1. As noted by LaSpada, a loss of wildtype androgen receptor, the protein that is mutated in SBMA, accelerates motor neuron degeneration and accentuates androgen insensitivity in a mouse model of SBMA. In addition, Aaron Bowman reported that the selective neuropathology of SCA1 is dependent on the association of mutant ataxin-1 with a subset of its native protein complexes. By generating mice expressing elevated levels of a paralogue of ataxin-1, ataxin-1B/Boat, Bowman and colleagues were able to suppress neuropathology in a mouse model of SCA1. The paralogue acts by displacing mutant ataxin-1 from the transcriptional complexes with which wildtype ataxin-1 normally interacts.

LaSpada concluded that polyglutamine diseases seem to share the same basic pathogenic pathways, however, their relative importance varies between diseases. To understand the uniqueness of each disease, he proposed focusing on protein context and, in particular, how distinct protein-protein interactions may account for specific cell vulnerability.

RNA-mediated pathogenesis in repeat disorders

A newly appreciated commonality between several repeat disorders which may have implications for HD is RNA-mediated pathogenesis and bidirectional transcription across repeat expansions. Participants described recent findings in myotonic dystrophy, spinocerebellar atrophy type 8 (SCA8), and Huntington's disease-like 2 (HDL2), and how they may relate to HD.

As described by Stephen Tapscott, many lines of evidence indicate that myotonic dystrophy is caused by a CUG expansion in the myotonic dystrophy protein kinase (DMPK) mRNA. The mutated mRNA causes muscle pathogenesis by sequestering the nuclear splicing factor muscleblind 1 (Mbn1). However, the expansion may have other effects at the DNA level. Recent findings indicate that normal, unexpanded CTG/CAG repeats in DNA can be components of transcriptional regulatory sequences known as insulators. Insulators block enhancers from interacting with promoters, an activity thought to help demarcate chromatin regions that are coordinately regulated. Although it is not entirely clear how insulators work, the process involves bidirectional transcription across repeat regions and an associated structural change in the local chromatin—euchromatin, usually associated with active transcription, is transformed into heterochromatin. The spreading of heterochromatin is constrained by the binding of insulator factors to specific sites flanking the repeats. Tapscott's recent studies of the myotonic dystrophy DM1 locus indicate its CTG repeats are components of an insulator. The repeats are flanked by binding sites for the insulator factor CTCF and Tapscott has detected small RNA fragments resulting from bidirectional transcription across the repeat region. In addition, the nucleosome associated with the repeat exhibits heterochromatin markers. Thus, it is possible that disruption of this normal function by repeat expansion may contribute to the myotonic dystrophy phenotype.

Another disease in which bidirectional transcription may play a previously unrecognized role is SCA8. As noted by Laura Ranum, the CTG expansion that causes SCA8 was originally identified as an apparently non-coding CTG expansion mutation and hence thought to involve an RNA gain-of-function mutation with a mechanism similar to that of the CTG expansion associated with myotonic dystrophy. Consistent with this proposal, Ranum observed molecular changes in the CNS involving CUG expansion transcripts interactions. However, as part of the characterization of a mouse model expressing the full-length human SCA8 gene, her team also observed intranuclear inclusions recognized by IC2, an anti-polyglutamine antibody. An in-depth examination of this surprising result revealed that bidirectional transcription of the SCA8 gene produces an antiparallel transcript with a CAG repeat that is translated to produce a polyglutamine protein. Thus, Ranum proposed that SCA8 pathogenesis is likely to be mediated by toxic gain-of-function mechanisms at both the RNA and protein levels.

Whether bidirectional transcription and/or RNA pathology contribute to HD is currently unknown. As illustrated by a large number of the meeting's presentations, there is much evidence indicating a prime role for huntingtin protein and polyglutamine expansion in HD. Furthermore, as noted by Kurt Fishbeck, observations of disorders such as SCA1 and Kennedy's disease further support the likely importance of polyglutamine toxicity in HD pathogenesis. Tapscott and Ranum acknowledged these points, but added that RNA pathogenesis may be an additional contributor to HD pathology.

Of particular interest, Tapscott noted his group has preliminary evidence of huntingtin antisense transcripts in the striatum.

Furthermore, he explained how these transcripts could be generated, and potentially affect HD pathology, even under conditions which may seem to preclude their production. For example, he noted that the insulator factor CTCF can bind various repeat sequences such that bidirectional transcription and insulator function could be occurring even in models of HD that have huntingtin constructs with mixed sequence repeats. In addition, antisense transcription could occur even in models with huntingtin cDNA transgenes lacking antisense promoters because of transgene concatamerization. Furthermore, although non-translated CAG repeats have been reported to be unable to cause disease, it is unknown whether stable antisense transcripts have been generated under these conditions.

Another reason to examine the possibility that RNA pathogenesis may contribute to HD is the observation from Russ Margolis's group that RNA toxicity seems to play a key role in the pathogenesis of Huntington's disease-like 2 (HDL2), a disorder whose pathology and symptoms are strikingly similar to those of HD. HDL2 has a repeat expansion within a variably spliced exon of junctophilin-3, but it is in the CTG orientation, not the glutamine-coding CAG orientation. Furthermore, Margolis's team was unable to find proteins bearing either polyglutamine, polyleucine or polyalanine expansions in HDL2 tissues, and junctophilin-3 knockout mice have only mild phenotypes. Based on these observations, Margolis searched for signs of RNA toxicity in HDL2 and discovered RNA foci in neurons similar to those described in myotonic dystrophy. The foci contain Mbn1 and junctophilin-3 transcripts that are not fully processed. Interestingly, protein inclusions are also observed in HDL2, but not in the same cells. Margolis suspects RNA foci are the prime mediators of HDL2 pathogenesis because junctophilin-3 constructs lacking translation initiation sites result in RNA foci and toxicity in cultured cells, which is partially rescued by Mbn1 overexpression. Taken together, the data suggest that further investigation into the potential contribution of RNA toxicity in HD is warranted.

Searching for therapeutic targets and compounds

The previously described data on HD's mechanisms of pathology suggest potential targets for drug development, as well as assays for drug screening. A wide range of potential intervention levels was discussed, ranging from targeting very early events in the disease process, such as the production of mutant huntingtin mRNA, to late downstream consequences, such as cell death. In addition, strategies for drug discovery ranged from high throughput screening of large libraries of compounds to the rational design of therapeutic molecules, and from those at the discovery stage to those involving clinical testing of candidate compounds.

Targeting mutant huntingtin mRNA and DNA

One of the conceptually most attractive options for treating HD is to target its seemingly primary source of pathology, the expression of the mutant huntingtin-encoding gene. The therapeutic potential of this strategy has been demonstrated by several recent studies, such as Beverly Davidson's report indicating that reducing mutant huntingtin mRNA with RNA interference (RNAi) prevents behavioral and neuropathological symptoms in HD mice. At the meeting, several participants described their efforts to optimize this strategy and, comparing these reports to those of 2004, it is evident that important progress has been made in the field.

A major challenge in devising an RNAi-based approach for treating HD is that it is impossible to selectively target the mutation underlying HD. Using RNAi against CAG repeats would affect both wildtype and mutant huntingtin alleles, as well as other genes with CAG repeats. One alternative is to design small interfering RNAs (siRNAs) that are huntingtin-specific, but which do not distinguish between mutant and wildtype mRNAs. Complete suppression of huntingtin is likely to be deleterious. As noted by Jodi McBride, previous studies by Reiner and colleagues suggest that normal huntingtin is critical for the survival of neurons in the adult forebrain. However, partial reduction of normal huntingtin may be tolerable. As noted in 2004, resolving this question is key to determining the feasibility of this approach.

This year, several participants presented encouraging findings suggesting that reducing wildtype huntingtin by RNAi is safe. For example, using species-specific small hairpin RNAs (shRNAs) against huntingtin in rodent models expressing human mutant huntingtin genes, Nicole Déglon's group was able to test the effects of downregulating wildtype and mutant huntingtin expression individually or jointly in the striatum. The results indicate that treatment with shRNAs that target mutant huntingtin specifically reduces HD-like pathology, and the effects are very similar to those achieved using shRNAs that target both wildtype and mutant huntingtin. Survival rates also appear to be similar. Thus, the downregulation of wildtype huntingtin does not seem to decrease the therapeutic efficacy of downregulating mutant huntingtin, nor induce any visible toxicity. So far, the team has followed the animals for 2–3 months after treatment, and they now plan to extend these observations to include later time points. Consistent with these findings, McBride developed and delivered an anti-huntingtin shRNA to mice striata using adeno-associated viruses (AAV) which downregulated huntingtin expression by 50–60% and caused no toxicity or behavioral defects.

Conducting safety studies in adult rhesus monkeys, Eric Burright's group has also seen no indications of adverse side-effects from wildtype huntingtin suppression. Burright developed AAVs carrying anti-huntingtin shRNAs to suppress huntingtin expression in the caudate and putamen. Quantitative PCR and laser-capture microdissection, revealed a 65–70% reduction in huntingtin mRNA. No gross neuroanatomical alterations, disruptions in the endoplasmic reticulum, significant changes in spontaneous activity, nor reductions in fine motor skills were observed. Some perivascular cuffing occurred, but it was related to viral delivery, not huntingtin suppression.

Without targeting a specific mutation, there are a very large number of possible sequences to target when designing huntingtin siRNAs, given the large size of huntingtin mRNA. Dinah Sah noted that her team at Alnylam has used FASTA analysis and proprietary algorithms to select sequences that are potent and selective in silencing huntingtin, and which share species homology between animal models and humans. Davidson added that trial-and-error is also a necessary component of siRNA selection. Her group has generated and experimentally tested siRNAs spanning all of huntingtin's exons, focusing on regions of inter-species homology. Illustrating the importance of experimental testing, McBride noted that two of three shRNAs developed by her group were toxic, resulting in reduced DARPP-32 staining, enlarged ventricles and microglial activation. The team suspects that inappropriate strand loading onto the RNA-induced silencing complex (RISC) may be causing the silencing of unintended mRNAs.

Although no obvious side-effects have been found associated with a partial reduction of wildtype huntingtin yet, it is possible that more subtle alterations will be discovered in the future. To avoid this

potential problem, Neil Aronin and colleagues are searching for heterozygotic single nucleotide polymorphisms (SNPs) to enable the production of siRNAs that distinguish between individuals' huntingtin alleles. In an analysis of 195 human brain samples, the team found 21 SNPs, 3 of which are novel. Many of these SNPs are amenable to RNA mismatch design. In addition, the team identified positions in siRNA molecules that confer high levels of discrimination between similar target mRNAs—e.g., mismatches at positions 10 and 16 in the guide strand seem to be particularly effective. Based on these findings, Aronin has created an siRNA that can knock-down the expression of a mutant huntingtin allele 10 to 20 times more effectively than that of a co-resident wildtype allele.

Although developing allele-specific treatments may be costly because of the multiple siRNAs that will need to be tested in clinical trials, several features make this approach appealing. Approximately 75% of the 195 samples Aronin analyzed displayed huntingtin SNP heterozygosity, suggesting that a large fraction of individuals carrying the huntingtin mutation could benefit from allele-specific therapy. Moreover, individuals with SNP heterozygosity usually have multiple heterozygous SNPs which could enable a highly effective downregulation of mutant huntingtin based on the combinatorial use of allele-specific siRNAs.

Participants also discussed technical issues regarding the stability of different types of silencers, as well as delivery modes and timing. Davidson noted her group has had success working with Sirna Therapeutics, a company that produces siRNAs with extensive chemical modifications and relies on lipid nanoparticles for delivery. Sah's team at Alnylam is using siRNAs that have several modifications, including phosphorothioate and 2'-O-Me chemical modifications, to protect them from exo- and endonucleases, and has also found that cholesterol-conjugation may boost efficiency.

The use of pumps and viral vectors to provide a continuous supply of silencing molecules was also discussed. Davidson noted that implanting a micro-osmotic pump in the striatum, but not the ventricles, resulted in excellent distribution of siRNAs, yielding a dose-dependent reduction in endogenous huntingtin reaching over 80%. The results suggest that pharmaceutical doses are feasible, but further safety and efficacy tests are needed. Viral delivery systems, such as lentiviral vectors and neurotrophic AAVs, were also mentioned. Advantages of these systems include the possibility of using reporters, such as GFP or LacZ, to track siRNA production, as well as generating siRNA constructs that can be conditionally expressed. Indeed, using conditional siRNA constructs, Déglon reported that siRNA therapeutic effects can be observed even when siRNA production is activated 2 months after the initiation of pathology in a rat model of HD. The finding is important because it suggests RNAi may be effective in symptomatic patients.

Alternatives to mainstream siRNA technologies were also discussed. Don Cleveland, for example, noted his group is using modified antisense DNA oligonucleotides, which rely on RNAse H activity for their effects, as therapeutic silencers. Using a pump to distribute these molecules throughout the CNS via the cerebrospinal fluid in rats and monkeys, Cleveland has achieved a 50% reduction in the expression of a mutated form of superoxide dismutase 1 which causes amyotrophic lateral sclerosis. Cleveland noted that, using a regulatable Medtronic pump, the approach is commercially feasible and safe, and his group expects to begin clinical trials next year. Applying the approach to HD, the team has been able to reduce huntingtin expression, and they now plan to test its efficacy in HD models.

An additional promising new approach was described by Kenneth Huffman, who presented his group's efforts at targeting chromosomal DNA to reduce gene expression. The advantages of

this approach include a low number of targets (only two per cell), the potential for target-specific mutation, and the availability of an alternative strategy to complement siRNA techniques. Huffman and colleagues have demonstrated the efficacy of antigene RNAs for various genes, including the progesterone receptor and huntingtin, by targeting the transcription site or upstream sequences of genes to block expression. Interestingly, antigenes appear to mediate their effects by promoting the association of Argonaute 1 and 2—proteins that are major components of the RISC implicated in RNAi—with the promoter of the antigene target. Thus, the argonaute proteins appear to link the silencing pathways that target mRNA with pathways mediating DNA recognition. Indeed, inhibiting the expression of the argonaute proteins reverses both transcriptional and post-transcriptional silencing.

Targeting mutant huntingtin protein

Interfering with mutant huntingtin at the post-translational level was also discussed as a therapeutic option. In 2004, Anne Messer described the development of intrabodies—single chain antibody fragments—to either alter the kinetics of mutant huntingtin misfolding or interfere with its toxic activities. The results presented were promising, but Messer noted a paucity of intrabodies capable of reducing aggregation and toxicity within a reasonable dose range.

This year, Amber Southwell working in Paul Patterson's lab reported the identification of new huntingtin intrabodies with improved efficacy. Previously, the team had developed several intrabodies to huntingtin exon 1, including MW7 which recognizes the huntingtin polyproline region, and reduces mutant huntingtin aggregation and toxicity in various models of HD. However, MW7 requires a 4:1 ratio of intrabody to antigen to mediate its effects. To generate more efficacious intrabodies to the polyproline region, Southwell screened a human synthetic phage display library of intrabodies using a peptide spanning a unique proline-rich sequence. Two new intrabodies, Happ1 and 3, were identified with better efficacy than MW7.

The most effective intrabody to mutant huntingtin reported to date is V_L12.3, an intrabody developed by David Colby and colleagues that recognizes the first 17 amino acids of huntingtin, and is optimally effective at a 1:1 ratio. The newly identified Happs are not quite as effective, but Happ3 can reduce toxicity and aggregation to a similar extent as V_L12.3, and the anti-proline intrabodies' mechanism of action differs from that of V_L12.3 in a way that may be therapeutically relevant. The Happs and MW7 reduce aggregated and soluble huntingtin, whereas V_L12.3 reduces aggregated huntingtin but increases diffuse huntingtin signal. Thus, Southwell proposed that the anti-proline intrabodies may be altering huntingtin turnover. The team is now using viral vectors to test the effects of Happs in animal models of HD.

Targeting mutant huntingtin's protein-protein interactions

Moving downstream from mutant huntingtin protein, several researchers have targeted polyglutamine aggregation. As described in 2004, Alex Kazantsev and colleagues, for example, used a high throughput yeast-based assay to screen 16,000 compounds for anti-aggregation activity. C2-8 emerged as a particularly potent anti-aggregation molecule, with an IC₅₀ of 50 nM in PC12 cells, and long-term inhibitory effects. The compound proved to be effective in PC12-based assays, R6/2 brain slice assays, and *Drosophila* toxicity assays.

This year, Vanita Chopra presented an evaluation of C2-8's therapeutic potential in R6/2 mice. Chopra noted that C2-8 crosses the blood-brain barrier and, when administered at 50 mg/kg twice a day for 5 days, can reach concentrations in the brain of 25 μM, well above its IC₅₀ in PC12 cells. Dose-escalation studies revealed that C2-8 is safe up to 200 mg/kg, twice a day. Chopra and colleagues also found that C2-8 ameliorates motor performance decline and decreases striatal nuclear aggregate volume. However, C2-8 had only a small effect on neuronal cell body volume, and no effect on aggregate density, striatal volume, or lifespan. The researchers will now test whether C2-8's effects can be improved by administering it before aggregation begins. Knock-in mice, which develop the disease more slowly, may be better models for these studies.

Participants also discussed the potential of other protein-protein interactions as targets for therapeutic intervention. In 2004, Hughes described using high throughput screens to identify huntingtin binding partners. This year, he summarized progress in validating a subset of these proteins as therapeutic targets. Hughes tested 60 of the 234 novel huntingtin-associated proteins his team identified for their ability to act as modifiers of neurodegeneration in a *Drosophila* model of HD. The screen revealed 48 genetic modifiers, many of which are loss-of-function suppressors and, thus, particularly amenable to drug development. In addition, 12 of the genes had both enhancer and suppressor alleles, suggesting potentially specific roles in HD pathogenesis. These genes encode several proteins related to synaptic function, including synaptic vesicle proteins, SNAP-25 and syntaxin 1a, as well as a calcium channel. Of particular interest, loss-of-function mutations in syntaxin 1a suppress eye degeneration, enhance survival, and ameliorate motor disruptions in a *Drosophila* model of HD. Recapitulating these findings, application of syntaxin 1a shRNA to a rodent brain slice exon-1 model of HD reduced neurodegeneration, while overexpressing syntaxin 1a enhanced the HD phenotype. Hughes proposed that HD is characterized by hyperactivity of a set of proteins involved in synaptic calcium influx, and by downregulating them, it is possible to rescue HD pathology. At least some of these targets, such as synaptic calcium channels, are druggable.

Targeting bioenergetic alterations

Based on the multiple studies indicating that bioenergetic alterations are an important part of HD pathology, some researchers are investigating the therapeutic potential of modifiers of energy balance. For example, as previously mentioned (see *Mitochondrial and bioenergetic alterations in HD*), Elisa Fossale and colleagues identified small molecules that increase ATP levels. These molecules are of particular therapeutic interest because ATP:ADP ratios are altered in HD, ATP deficits occur early in the disease process, and the deficits are dependent on CAG repeat length.

Creatine is another bioenergetic compound with therapeutic potential which is already being tested in clinical trials. As described by Steve Hersch, creatine is a putative neuroprotective agent with various functions. It buffers ATP levels, acts as an antioxidant, reduces glutamate release, and potentially stabilizes the mitochondrial permeability transition (MPT). Initial clinical studies evaluating primarily creatine's safety and its effects on HD showed that 3 to 10 grams of creatine a day are safe and tolerable. The study was unable to demonstrate symptomatic relief because, as a safety study, there were not many subjects enrolled. The Creatine Therapy for HD (CREST) study, for example, tested the effects of 8 g/day of creatine for 4 months. Although it revealed no clinical effects, it induced partial suppression of 8-OH²dG, a

marker of DNA oxidation that is increased up to four-fold in HD plasma.

A subsequent dose escalation study yielded more encouraging results. First, it revealed that using higher doses of creatine is feasible. Although doses above 35g/day cause gastrointestinal problems and no increased clinical benefit, lower doses appear to have no ill effects. Serum creatine levels plateau at 35g/day and then start decreasing. In the frontal cortex, creatine levels continue to increase, at least up to 40 g/day. The new studies also revealed that high doses of creatine appear to have therapeutic effects: 20–30 g/day of creatine almost completely normalized 8-OH2'dG levels, inhibited cortical thinning associated with HD (see **HD Biomarkers**), and improved cognitive function. To confirm and extend these observations, which include only 10 individuals, Hersch plans to conduct a three-year study with 650 subjects using creatine at 30g/day.

Targeting cell-cell interactions

As previously noted, there is increasing evidence that cell-cell interactions play a key role in HD pathogenesis. Thus, modulating these interactions could be of therapeutic value. For example, Tie-Shan Tang's results indicating that dopamine potentiates glutamate-induced apoptosis in a cellular model of HD, suggest that downregulating dopamine may have beneficial effects. Tang noted that, in addition to tetrabenazine's ability to control the debilitating chorea associated with HD, as described at the 2004 meeting, tetrabenazine may act as a neuroprotective agent. However, the timing of administration may be crucial. According to one participant, HD patients in Europe who have been exposed to high doses of neuroleptics for decades have not shown signs of improvement in disease progression. Tang hypothesized that these patients probably did not receive the drugs early enough to benefit from their potentially neuroprotective effects.

Cystamine was also suggested as a compound that may help normalize cell-cell interactions. Cystamine was originally thought to ameliorate HD pathology by acting as a transglutaminase inhibitor to reduce mutant huntingtin aggregation. This explanation had to be revised, however, when cystamine was found to be effective in mice lacking the tissue transglutaminase gene. Cystamine has multiple effects on cells in addition to inhibiting transglutaminase activity: it inhibits caspase activity, increases antioxidant levels and, as most recently described by Sandrine Humbert, increases BDNF secretion. Humbert and colleagues proposed this latter function is at least partially responsible for cystamine's beneficial effects in HD. Cysteamine is an FDA-approved drug which increases serum levels of BDNF in mouse and primate models of HD. Cysteamine is a reduced form of cystamine which is also neuroprotective and increases BDNF levels in the HD mouse brains. Cystamine is not protective, however, in HD mice with decreased levels of BDNF. Humbert concluded that cysteamine, or cysteamine analogs, are promising drug candidates for HD, whose effects could be readily monitored in blood samples.

Targeting cell loss

Although the contribution of cell loss to HD pathology remains unclear, encouraging results were presented for therapeutic candidates that inhibit cell death, stimulate neurogenesis, or replace lost neurons. For example, Sue Browne reported an amelioration of several HD-associated alterations in response to delivery of the anti-apoptotic factor, XIAP, by gene therapy. Browne's team used

adeno-associated viruses to deliver a truncated form of XIAP lacking a domain required for proteolysis, the RING domain, to cell and animal models of HD. The construct protected cultured cells against polyglutamine toxicity. In addition, when injected into the striata of N171-82Q mice at 8 weeks of age, approximately 3 weeks before symptom onset, it nearly normalized rotarod performance. However, weight loss was unabated and the effects on lifespan were moderate (16%). To determine how XIAP is mediating its beneficial effects, Browne and colleagues have created several XIAP mutants that affect XIAP's ability to block caspases 3, 7, and 9 or sequester the pro-apoptotic mitochondrial protein Smac/Diablo. So far, Browne's results indicate that XIAP's caspase domains are not required for neuroprotection in vitro, whereas the Smac/Diablo domain appears to be important.

Promoting neurogenesis was also discussed as a therapeutic option to treat HD. Abdellatif Benraiss noted that neural stem cells persist throughout the ventricular subependyma of the adult vertebrate brain, and these cells can be stimulated to produce neurons in response to BDNF overexpression. In addition, the process can be potentiated by adding noggin, an inhibitor of the bone morphogenetic protein (BMP) which induces glial cell production. To test the therapeutic potential of these effects in HD, Benraiss and colleagues treated 4-week-old R6/2 mice with adenoviruses overexpressing BDNF and noggin. The treatment resulted in striatal neuron recruitment, delayed motor impairment-including improved open field volitional activity and a slower decline in rotarod performance-and extended survival. Injection of a mitotic inhibitor abrogated these effects, confirming they were mediated by the induction of neurogenesis.

Another strategy to replace lost or dysfunctional cells in HD is cell transplantation. As summarized by Anne-Catherine Bachoud-Lévi and Stephen Dunnett, HD is a good candidate for cell transplantation because it is a progressive and untreatable disease that can be unambiguously diagnosed. In addition, there are good animal models of striatal repair which have allowed the development of transplantation techniques. Studies in Parkinson's disease have demonstrated that striatal grafts can survive, grow, and exert useful clinical effects. As noted by Dunnett, however, there is still only limited data on the effectiveness of transplantation in HD. Clinical studies have been performed since the late '80s, but only a few dozen patients have actually undergone the procedure and, of those, only 3 have shown clear signs of improvement.

The most promising pilot study was conducted by Bachoud-Lévi's group, who implanted striatal neural precursors and neuroblasts into the striata of 5 HD patients. Four of the patients showed motor and cognitive improvements, which correlated with enhanced metabolic activity of the transplants and the frontal cortical areas connected to them, as assessed by MRI and PET imaging. The improvements lasted several years in 3 of the patients, with a slow decline starting 3–6 years after surgery. Cognitive function remained close to pre-operative values for 8 years post-operatively. Chorea remained reduced but dystonia slowly increased post-surgery. All patients are still living up to 12 years following the transplantation. To extend these studies, a controlled, randomized, multicenter trial with 60 patients has been organized. The trial is still in its early stages, but so far the surgeries are going well. Only a few post-operative complications have been reported and, except for one case of hematoma, patients have recovered fully.

To refine and optimize translation, there are still several technical and theoretical issues that must be addressed, however. For example, many uncertainties are associated with fetal tissue preparation—including the optimal selection of donor age, tissue dissection and

cell preparation. In addition, more studies on implantation procedures, including tissue placement and immunosuppression protocols, are needed. Several aspects of patient selection and trial design also remain to be scrutinized. As noted by Dunnett, the Core Assessment Program for Intracerebral Transplantation (CAPIT) protocol promises to be useful for conducting reliable comparisons between studies.

At a theoretical level, Dunnett noted that it is still unclear if the many observations implicating extra-striatal pathology make striatal repair irrelevant. Even if striatal repair is beneficial, it is uncertain how long its benefits will last. The effects of disease progression are also unknown. Will cells implanted early in the disease process fare better than those implanted later? Will transplants ultimately succumb to the disease process? Both Bachoud-Lévi and Dunnett noted that coupling transplantation with a neuroprotective treatment will probably enhance outcomes. Indeed, the decline observed in Bachoud-Lévi's pilot study suggests that neurotrophic factors or agents that interfere with cell death might act synergistically and help prolong the benefits of transplantation. The graft itself could provide a vector for delivery of these factors.

Another issue that needs further exploration is the source of cells for transplantation. As noted by Dunnett, primary fetal cells, xenografts, or stem cells can be used. Of particular interest, Elena Cattaneo and colleagues have recently developed neural stem cells derived from embryonic stem (ES) cells, as well as from fetal and adult brain tissues. As described below (see *Looking ahead: Tools that promise to advance HD research*), these cells are stable, homogenous, and retain their neurogenic capacity even after multiple passages *in vitro*. The team is currently characterizing differentiated cells that have been transplanted into mice to assess their functions *in vivo*. Although most of the work has been performed in mice, the researchers are now optimizing conditions to obtain neural stem cells from human ES cells.

In sum, promising data have emerged from transplantation studies in HD, but the approach is still in its early stages of development. Dunnett considered that many of the open questions will be best resolved by conducting small clinical trials to achieve a step-by-step refinement of the procedure. Although animal models may help, direct testing in humans will be key.

Targeting pathways that regulate aging and metabolism

Tapping into endogenous protective pathways may also be fruitful. In particular, Leonard Guarente suggested that targeting sirtuin proteins, nicotinamide adenine nucleotide (NAD)-dependent histone deacetylases (HDACs), may have therapeutic potential for the treatment of many diseases, including HD. Studies in various animals have shown that enhancing the activities of the sirtuin protein homologues Sir2 (in yeast) and Sirt1 (in mammals) increases both replicative and non-mitotic lifespan. Sir2/Sirt1 has been shown to mediate the life-expanding effects of caloric restriction, suggesting it may be involved in linking aging and metabolism. Consistent with this possibility, Sirt1 plays a key role in the transcriptional regulation of various metabolic pathways and stress responses, including insulin production, inflammation, and axonal protection against injury. Transgenic mice overexpressing Sirt1 in all cells have lower body weights, less white adipose tissue, lower cholesterol levels, and lower insulin and glucose levels. As noted by Guarente, Sir2/Sirt1 may be a key player in an evolutionarily adaptive mechanism which involves the slowing down of metabolic functions when food is scarce.

To test its potential as a therapeutic target for HD, Guarente and colleagues are crossing transgenic mice which over- or under-

express Sirt1 with R6/2 mice. In addition, Linda Kaltenbach and colleagues are examining the effects of over-expressing or silencing various sirtuins in a brain slice model of HD. These endeavors are of particular interest in light of previous findings presented at the 2004 meeting by Alex Parker from Christian Neri's group. Parker reported the identification of Sir2 as a genetic modifier of polyglutamine toxicity, noting that Sir2 activation reduces dystrophic axons and neuronal cell dysfunction, while Sir2 inactivation potentiates cytotoxicity. In addition, in 2005, Parker and co-workers published data indicating that the sirtuin activator resveratrol prevents polyglutamine-induced toxicity in *C. elegans* and in cultured HD mouse neurons.

It is also worth noting that several recent studies indicate that HD pathology is reduced by HDAC inhibitors. To explain this apparent contradiction, Guarente noted that sirtuins are merely one of several classes of HDACs with unique substrate specificities. Thus, broad-spectrum inhibitors can have multiple effects, and probably ameliorate HD toxicity through the inhibition of non-sirtuin deacetylases.

HD Biomarkers

Testing the therapeutic potential of the above mentioned candidates in an efficient manner will greatly depend on having reproducible, sensitive and specific biomarkers of disease progression. In 2004, participants considered the identification of such indicators a top priority in HD research. This year, new biochemical markers, cognitive tests and brain imaging measurements emerged as potential candidates. Of particular interest, the first use of a biomarker to accelerate phase II clinical studies was presented, as well as the identification of markers that reliably track presymptomatic disease progression.

Based on the known transcriptional disruptions associated with HD and huntingtin's ubiquitous expression throughout the body, Dmitri Krainc's group searched for HD-associated changes in gene expression in peripheral blood. Using oligonucleotide microarrays to analyze global gene expression, Fran Borovecki and colleagues identified 322 mRNAs that were significantly altered in HD blood samples. Selecting a subset of 12 genes whose expression was well correlated with HD, the team was able to distinguish controls, presymptomatic individuals, and symptomatic HD patients. The transcriptional alterations were not observed in patients with other disorders, including Parkinson's disease. The results have now been validated in a new set of 50 HD patients and 6 genes have been selected as particularly robust indicators of HD progression. Principal components analysis revealed that early presymptomatic individuals have mRNA profiles similar to those of controls, whereas late presymptomatic individuals have expression patterns that more closely resemble those of symptomatic patients.

In addition to this statistical approach, Borovecki and colleagues are using Gene Set Enrichment Analysis (GSEA) to identify biological pathways that are disrupted in HD. The results have revealed alterations in glucose metabolism and proteasome function. Borovecki now hopes to identify additional candidate genes and evaluate the effects of drug treatments on expression patterns. In addition, the team is conducting studies in R6/2 and *Hdh* knock-in mice. So far, GSEA indicates that RNA processing, translational regulation, mitochondrial function, and caspase pathways are altered in these HD models. Borovecki concluded that gene expression patterns hold promise for acting as biomarkers of HD. Moreover, he emphasized the value of using large sample numbers and multiple markers to obtain reliable signatures of disease.

Another ‘omic’ approach to identify HD biomarkers was described by Lisa Paige. She and her colleagues are analyzing the plasma of HD patients and controls to identify metabolic alterations that track HD progression. The pilot study includes 69 subjects whose plasma samples have been extracted and subjected to both liquid and gas chromatography followed by mass spectrometry. Surprisingly, several of the HD-associated changes detected to date are gender specific. For example, alanine and isoleucine decrease with HD progression, but only in females. Two metabolites that form a redox couple, bilirubin and biliverdin, increase in females with HD, but decrease in males, suggesting the genders may deal with oxidative stress in different ways. Moreover, whereas salicylic and salicyluric acids increase with HD progression in males, they remain unchanged in females. Salicylic and salicyluric acids are found in vegetables and processed foods and are normally eliminated by the body. The significance of all these differences remains unclear, but Paige highlighted the importance of taking gender into account when searching for biomarkers of disease.

In addition to global searches for biomarkers, specific candidates are being assessed for their biomarker potential. Maria Björkvist and colleagues, for example, are examining the levels of various neuropeptides in the cerebrospinal fluid (CSF) and serum of HD patients. HD patients suffer from several symptoms—such as depression, sleep problems, and profound weight loss—that suggest neuroendocrine dysfunction. Indeed, in 2004, Åsa Petersén reported that orexin-producing cells of the lateral hypothalamus are progressively lost in R6/2 mice and orexin levels are correspondingly reduced in the CSF. Although subsequent studies in humans showed no detectable changes in CSF orexin levels associated with HD, other observations suggested that additional neuroendocrine peptides might be altered. As noted by Björkvist, food intake is controlled by several peptides and the weight loss associated with HD in the mice can’t be explained by orexin loss alone. Furthermore, in 2004, Karen Reue noted an altered relationship between leptin levels and adipose tissue mass in two HD mouse models. To test whether the levels of various peptides that control food intake are altered in HD, Björkvist has now analyzed CSF and serum samples from controls and HD patients. The data indicate that HD is associated with increases in neuropeptide Y, and cocaine-amphetamine regulating transcript (CART) in CSF, and ghrelin and interleukin-6 in serum. Furthermore, leptin is decreased in serum. The data help advance the understanding of weight loss in HD, and provide new candidates for biomarker development.

Another indicator of hypothalamic dysfunction which may be useful as a biomarker was presented by Nigel Woods. Reasoning that the regulation of drinking might be abnormal in HD due to alterations in hypothalamic function, Woods and colleagues used the LABORAS behavioral monitoring system to examine drinking in R6/2 mice. They observed that at approximately 10 weeks of age, R6/2 mice start eating and drinking more than wildtype mice, a behavior that persists until 18 weeks of age. The increase does not appear to be due to a generalized increase in locomotor activity. As diabetes occurs later in these mice, it would appear that the increase is also not due to the development of diabetes. To extend these observations, Woods gave HD patients a xerostomia (dry mouth) questionnaire. Consistent with his observations in mice, the scores were much higher in HD patients than controls, and UHDRS scores correlated with xerostomia scores. In addition to providing a new candidate biomarker, the data suggest that dry mouth may contribute to weight loss in HD by inter-

fering with tasting, chewing and/or swallowing. Woods added that these findings should be taken into consideration when using oral administration to deliver drugs in mice.

Participants also discussed the potential of cognitive tests as biomarkers. One effort that is importantly contributing to the development of such tests is the PREDICT-HD study led by Jane Paulsen. As described by Sarah Queller, PREDICT-HD is a multi-center, longitudinal, prospective study which aims to establish predictors of HD diagnosis, and refine measures that track disease progression for conducting preventive clinical trials. The study includes members of HD families who have been genetically tested and know their test results. The majority of participants have been found to have expanded CAG alleles with the minority containing normal CAG alleles as controls. There are 594 current, and 900 expected, participants. The study is tracking cognitive, motor, psychiatric and biological changes that occur in presymptomatic individuals. Reporting on the predictive value of cognitive tests, Queller noted that 16 of 17 cognitive and psychomotor tests are significantly associated with estimates of years to clinical onset (based on age and CAG repeats). The tests measured a variety of abilities, including finger tapping skills, timing and movement selection, executive functions, learning and memory, face and emotion recognition, as well as odor identification. Thus, cognitive markers are promising as clinical markers for progression of pre-diagnostic HD pathology. As these are only estimates, it remains to be seen how well these measures will actually predict as individuals are followed longitudinally.

Magnetic resonance imaging of the brain is also emerging as a particularly valuable source of pre- and post-diagnostic HD biomarkers. As noted by Diana Rosas, HD is a disease with widespread effects in the brain that can be detected with imaging technologies. At the 2004 meeting, Christopher Ross described early changes in striatal volume and white matter appearance, as well as changes in brain activity patterns. This year, Rosas discussed new findings, including the use of cortical thinning as a biomarker for a phase II clinical trial.

Rosas first noted that, to some extent, cortical and striatal changes are independent of each other, with cortical changes being more closely correlated with clinical parameters. Regional cortical thinning begins before clinical onset, but its levels reach only 20–30% at onset, whereas striatal volume is already reduced by 50% at clinical onset. Of particular interest, cortical thinning is well correlated with performance on several cognitive tests. Cortical thinning is progressive, with measurable changes occurring in as little as a year and involving more cortical regions over time. During the time prior to observable clinical symptoms, signs and diagnosis, primary motor and sensory areas are significantly thinned paralleling transcriptional data obtained from microarrays from postmortem tissue. Over time, more of the cortex undergoes atrophy, and extends to involve regions of parietal, occipital and eventually frontal cortex.

To test cortical thinning’s potential as a biomarker of HD, Rosas used this measure in a Phase II study of creatine described above (see *Targeting bioenergetic alterations*) led by Steve Hersch. Mirroring creatine’s beneficial effects as assessed by cognitive tests and reduced 8-OH²dG serum concentrations was significantly reduced in response to creatine treatment. Creatine even provided protection against thinning of the cortex. As noted by Hersch, the use of this novel biomarker promises to dramatically reduce the duration of clinical trials.

Looking ahead: Tools that promise to advance HD research

Monitoring cell fates

Participants described several tools that promise to further the understanding of HD's mechanisms of pathology, and help discover new drug targets and candidate therapeutic compounds. For example, Steve Finkbeiner presented a strategy for identifying and monitoring cellular phenotypes which should help sort the myriad alterations observed in HD into causal relationships, compensatory changes, and epiphenomena. As noted by Finkbeiner, commonly used "snap-shot" assays, in which the initial step involves grinding up tissues or cells, fail to provide longitudinal and cell-specific information which is often key to the understanding of pathogenic mechanisms. They are prone to detection biases because short-lived phenomena are more difficult to detect than long-lived phenomena. In addition, they do not account for cell heterogeneity. These considerations are particularly important when studying neurodegeneration, a slow, cell-selective, and stochastic process in which key pathological stages can be short-lived, and where primary disruptions, incidental changes, and cellular compensatory mechanisms can be hard to distinguish.

Finkbeiner's approach automatically monitors the fates of thousands of cells over time and correlates the risk of death or dysfunction with other cellular parameters. The system relies on an automated microscope that returns to the same cell after defined intervals. Computer programs collect images, and define and count objects in different categories. The system allows researchers to obtain data from a million cells in 10 minutes, as well as track individual cells across several months. It is more stable and quantitative than conventional assays, and 100 to 1000 times more sensitive. Applying modified survival analysis methods, Finkbeiner's team can determine whether and how factors measured during longitudinal analysis predict a particular biological outcome.

For example, in 2004, Finkbeiner published a study using striatal cells expressing GFP-labelled huntingtin constructs showing that the amount of diffuse intracellular huntingtin predicts whether and when inclusion body formation or death will occur. In addition, the study revealed that inclusion body formation predicts improved survival and leads to decreased levels of mutant huntingtin elsewhere in a neuron. The team is now extending these studies using cells expressing the GFP constructs under cell type-specific promoters. Moreover, they are testing sensitive cameras to monitor cells whose constructs are expressed at low levels, closer to those of endogenous huntingtin.

Finkbeiner's system also allows multiplexing. Processes such as inclusion formation, proteasome function, transcription, autophagy and synaptic function can be monitored with fluorescent assays. And using spectral imaging to separate overlapping fluorescent emissions, the team can simultaneously resolve more than 10 fluorescent tags and quantify co-variables to study disease mechanisms and drug effects from a systems biology perspective. Future plans also include conducting high throughput drug screening, as well as adapting the system to perform two-photon, single-cell imaging in living mice.

New HD models

Addressing a need noted in 2004, participants also presented new HD models for investigating disease mechanisms, as well as for drug discovery. As previously described, William Yang's lab has

generated the BACHD model in which mutant huntingtin can be selectively switched off in specific cell types. Mice in which mutant huntingtin is downregulated in all neurons have been generated, as well as mice in which mutant huntingtin is downregulated exclusively in the cortex. Future plans include the suppression of mutant huntingtin in striatal medium spiny neurons, cortical parvalbumin interneurons, astrocytes or microglia.

Moreover, Michelle Gray pointed out that BACHD mice expressing the transgene in all of their cells provide a new model for HD that shares many similarities with human, adult-onset HD. The mice have progressive motor alterations as assessed by rotarod performance, and late-onset (12 months) hyperactivity. In addition, they exhibit selective neuropathology at 12 months, which includes the cortex and striatum, but not the cerebellum. Dark neuron degeneration is observed at this time, and aggregates can be seen in the neuropil at 12 and 18 months of age, mostly in the cortex. Interestingly, neither progressive nuclear translocation nor accumulation of fragments of mutant huntingtin are detectable at either 2 or 12 months of age, suggesting these processes are not required for pathogenesis, at least under these conditions. As previously mentioned, another new mouse model of HD was described by Michelle Ehrlich. These mice express a fragment or full-length mutant huntingtin predominantly in the medium spiny neurons of the striatum under the control of "striatal-specific" fragments of the DARPP-32 gene.

A model that is particularly well-suited for target validation and drug discovery was described by Linda Kaltenbach. Kaltenbach used a striatal brain slice model of HD developed by Don Lo and colleagues to validate candidates that have emerged from large-scale proteomic screens for proteins that interact with huntingtin. The system relies on transfecting striatal rat brain slices with various constructs simultaneously, using biolistic acceleration of gold particles, a method which results in nearly 100% co-transfection linkage. Co-transfection of a mutant huntingtin exon 1 construct and yellow fluorescent protein (YFP), allows the visualization of transfected cells. Labeled cells die early, and exhibit intracellular inclusions, dysmorphic dendrites, and altered electrical properties prior to death. To evaluate target candidates, Kaltenbach co-transfects the mutant huntingtin construct and YFP marker with plasmids coding for short hairpin RNAs to silence a target gene, or plasmids containing CMV-driven cDNAs to overexpress a target gene.

Important advances in the generation of cell models of HD were also presented. As noted by Elena Cattaneo, several cell models of HD already exist (e.g., ST14A cells that overexpress huntingtin, ST14A cells with an inducible copy of huntingtin, or Hdh knock-in cells). However, immortalized cells have alterations in their differentiation patterns which can affect their behaviors, and some cells are difficult to obtain in large amounts. In 2004, participants noted that the creation of new cell models would be particularly valuable for future research. Addressing this need, Cattaneo's team has developed and characterized neural stem cells derived from embryonic stem (ES) cells, as well as from physiologically relevant brain tissues derived from both fetuses and adults. These cells are stable, homogenous, and grow in a monolayer in serum-free medium. Moreover, they retain their neurogenic capacity even after multiple passages *in vitro* (170 passages over the course of 2 years). ES-derived neural stem cells express neural stem markers, but not markers for ES cells, mature neurons, or glial cells. By optimizing the conditions for *in vitro* differentiation, the team has now achieved high survival rates, 80% neuronal differentiation resulting in 90% GAD67- and GABA-positive cells, development of action potentials by 14 days, and responsiveness to GABA or glutamate agonists. The

cells express calcium and sodium channels, as well as NMDA and GABA receptors.

Currently, Cattaneo's team is characterizing neural stem cells that stably overexpress wildtype or mutant huntingtin. They appear to grow well, but exhibit increased sensitivity to the neurotoxin 3-nitropropionic acid which is ameliorated by BDNF. In addition, the team is deriving neural stem cells from R6/2 mice. They are also optimizing conditions to obtain neural stem cells from human ES cells, and characterizing differentiated cells that have been transplanted into mice to assess their function *in vivo*.

New clinical resources

New clinical resources also promise to help advance HD research. In particular, Rick Myers described COHORT, a research project of the Huntington Study Group which will provide scientists with prospectively collected clinical data and biological specimens. COHORT's aims were briefly described in 2004, however, the project was still in an organizational phase. Now, the first 137 participants have been enrolled. In the long-term, thousands of individuals are expected to participate, including people with symptomatic Huntington's disease, at-risk but untested individuals, individuals who on testing are found to have an expanded HD allele but, as yet, are undiagnosed, and family members with no risk for HD. Each participant will have an annual study visit, which will include the collection of clinical data, family history information (optional), and biological specimens (optional). Baseline demographics, medical history, results from physical and neurological examinations (including the UHDRS), and information on the use of medications will be collected, as well as DNA, plasma, urine, cell lines, CSF, if possible, and lymphocytes. Myers stressed that COHORT organizers intend to make the data and samples as widely available as possible, and urged participants to visit www.huntington-study-group.org and www.huntingtonproject.org for more information.

Participants also discussed the development of a new tool for the management of HD. Sir Michael Rawlins presented efforts led by the Hereditary Disease Foundation and the European HD Network to develop international, evidence-based guidelines to inform and improve the clinical management of HD. As the Chairman of the UK's National Institute for Health and Clinical Excellence (NICE) and a practicing clinical pharmacologist, Sir Michael has had decades of experience in developing evidence-based practice guidelines. Rawlins noted that the development of the HD guidelines will

include: identification of stakeholders, delineation of the scope of the guidelines, recruitment of members of the guideline development group(s), systematic review of published and unpublished data, and dissemination of the guidelines. To avoid the common pitfall of working with an overly ambitious scope, the guidelines will be initially limited to the management of symptomatic, adult-onset HD. A separate group is planned to develop evidence-based guidelines for treating juvenile HD. Currently, a review of data for treating adults is underway to assess the effectiveness of different treatments and identify unresolved questions. Small studies and variability between the measures used in different studies make it difficult to reach definitive conclusions, but organization of the data into hierarchies of evidence should help the development of the recommendations. Rawlins noted that the process is arduous, will probably take two years to complete, and will then require annual updates, but it should enhance patient care significantly.

Concluding thoughts

In sum, the meeting illustrated the significant progress that HD research has made in the past two years, and suggested a promising future with new tools and resources to tackle HD. With its broad scope, it reminded participants of the surprising complexity of a disease caused by a single, dominant mutation. Thirteen years after the discovery of the huntingtin gene, HD continues to provide new puzzles and surprises. The potential involvement of RNA pathogenesis, for example, and the apparent gender-associated differences in the body's responses to HD, stimulated new ways of thinking about the disease. Despite these continuing surprises, the development of therapies seems to be advancing rapidly. The convergence of data on BDNF and synaptic transmission as promising therapeutic targets, for example, is encouraging, as are the advances in RNAi technologies, and the early results of high doses of creatine in clinical trials. Furthermore, new tools and resources, such as neural stem cells and an automated microscope system to determine factors that predict neuronal fate and the emerging surrogate markers of HD pathogenesis, suggest major advances to come. It is impossible to predict the path that lies ahead in what has been, so far, a long and winding trajectory. But if the pace of discovery and development seen in the past two years persists, it is not unreasonable to expect that by the next meeting in 2008, the HD community will have made even greater strides in transforming HD into a treatable and curable disease.