

The HD Therapeutic Pipeline

January 7-8, 2006
Santa Monica, CA

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Abstract

The Huntington's disease (HD) therapeutic pipeline boasts a rapidly growing number of therapeutic candidates ranging from compounds in the initial phases of experimental testing to drugs being evaluated in human clinical trials. Participants at the workshop discussed the status of these candidates, highlighting promising approaches, identifying potential limitations, and providing ideas for future directions.

Targeting the primary source of HD pathology using RNAi to reduce the expression of mutant huntingtin was considered a particularly promising approach. However, participants were also enthusiastic about more general approaches targeting cellular pathways that have been implicated in various neurodegenerative disorders. For example, ways to modulate Nrf2—a master regulator of cells' defense system against oxidative stress—and SIRT1—a histone deacetylase implicated in longevity—were discussed. In addition, participants analyzed therapeutic opportunities for regulating heat shock protein networks and the search for inhibitors of toxic protein aggregate forms. Targeting mitochondrial dysfunction and finding ways to enhance cells' endogenous clearance mechanisms were also considered. Participants agreed that studying general protective pathways can provide, not only promising neuroprotective targets, but handles for the subsequent identification of more specific HD targets.

In addition, participants discussed the challenges of translating basic scientific findings into clinical successes. Noting a disconnect between basic research efforts and clinical projects, they agreed that improving communication between the two groups is sorely needed. Clinically relevant information, such as dosages, is often lacking, as well as validation criteria to guide the selection of candidates for clinical trials. There is also a paucity of reliable biomarkers of disease progression. Participants agreed that these are exciting times for HD research, with several promising therapeutic candidates on the horizon, therefore, it is time to address these translational issues more vigorously.

Introduction

Thirteen years ago, researchers took the first major step in the battle against HD by pinpointing its underlying mutation. The single dominant mutation—a CAG expansion in the gene encoding “Interesting Transcript 15” (IT15), now known as huntingtin—provided the key to begin understanding the cellular and molecular underpinnings of HD and designing ways to treat it.

As illustrated by a woman suffering from HD who generously shared her experiences with workshop participants, the discovery has already helped HD families. People at risk for the disease can choose to be tested for the mutation, which can help them plan their lives. Couples can decide whether or not to have children, for example, or decide to have their embryos tested before birth. The woman said she felt fortunate that she and her eldest son were able to find out if they carried the mutation, and she hopes her younger son will choose to be tested as well. Moreover, there have been improvements in the management of HD associated symptoms. The woman compared her situation to her mother's, who died of HD, and noted she has more options, some of which have helped her significantly, such as use of the selective serotonin reuptake inhibitor Celexa.

The challenge now is to move beyond these important, but mitigative, clinical victories and deliver effective HD treatments. Several promising therapeutic candidates are moving through the HD pipeline, but it is difficult to predict how and when they will yield definitive

clinical solutions. Participants at the workshop brainstormed ways to optimize and, when possible, accelerate this process. They discussed the status of some of the most promising candidates, identified hurdles to overcome, and proposed new strategies to speed therapeutic development.

A primary approach: targeting mutant huntingtin with RNAi

Current status

One of the most conceptually attractive options for treating HD is to target its primary source of pathology, mutant huntingtin. Using RNAi to reduce huntingtin mRNA levels, Beverly Davidson and colleagues are pursuing this approach. They are developing techniques that decrease both wildtype and mutant huntingtin RNA, as well as techniques that target the mutant allele specifically.

Davidson noted that partially reducing mutant huntingtin expression can improve symptoms in a mouse model of HD (Harper et al, PNAS, 2005). Her team injected into the striata of HD-N171-82Q mice adeno-associated virus AAV1 carrying a small hairpin RNA (shRNA) that knocks down expression of the human mutant transgene by approximately 60% without affecting normal mouse huntingtin expression. Both behavioral symptoms and neuropathological abnormalities improved significantly.

An open question, however, is whether neurons can tolerate and benefit from a partial nonselective knockdown of *both* normal and mutant alleles as would occur if a similar strategy were applied to humans. To address this question, Davidson's team has analyzed all huntingtin exons to find and rate useful RNAi targets that are conserved in mouse and humans. They currently have three top candidates which they plan to test in mice, and eventually in non-human primates. To deliver the shRNAs, they are using AAV, as well as non-viral delivery systems, and have generated GFP constructs to monitor vector delivery. In addition to the experimental shRNAs, they will test the effects of delivering vector alone and vector carrying mismatched shRNAs.

To assess the effects of these treatments, the group will monitor behavioral symptoms and neuropathological alterations. Brain tissue will be examined using hematoxylin-eosin staining, Nissl staining, inflammatory markers, TUNEL staining, and markers of astrocyte and microglial activation. Davidson estimates that by the end of the year they will know whether the approach is worth pursuing.

Davidson's group, as well as Neal Aronin's group, have also been working on allele-specific approaches to selectively knock down mutant huntingtin expression. Davidson's PNAS study, in which the human huntingtin transgene was knocked down without affecting the expression of endogenous mouse alleles, indicates that allele-specific silencing can be accomplished *in vivo*. The researchers are now targeting an HD-linked polymorphism identified in exon 58 and searching for more candidate polymorphisms by analyzing published human SNP data and scanning huntingtin sequences one nucleotide at a time. To monitor the activities of the shRNAs they develop, they have generated fluorescently labelled, full-length cDNA constructs of wildtype and mutant human huntingtin alleles, each harboring corresponding wildtype- or disease-associated SNPs.

Challenges and open questions

Participants discussed the challenges that lie ahead and offered suggestions to overcome them. For example, Carl Johnson noted that if decreasing wildtype huntingtin levels has deleterious effects that outweigh the benefits of reducing mutant huntingtin, delivering a shRNA-resistant copy of wildtype huntingtin together with the therapeutic shRNA might be worth testing. Stefan Kochanek and colleagues are pursuing this approach using “gutless” adenovirus vectors which lack parts of the viral genome and can thus carry large pieces of heterologous DNA.

Key to deciding which RNAi therapies are worth pursuing clinically will be the outcome measures used to evaluate them. Chesselet worried that these measures might overlook significant effects, such as deleterious consequences caused by reducing wildtype huntingtin. Although she considered Davidson’s neuropathological and behavioral tests robust, she noted they do not directly reflect important aspects of cellular function that could be disrupted, such as synaptic function. Chesselet urged participants to think carefully about the depth of analysis that should be required to consider a particular procedure sufficiently safe and beneficial to warrant testing in humans.

Participants also discussed various delivery issues. Chesselet pointed out that it is very important to use an appropriate viral serotype to ensure delivery into the desired cell types. Davidson said that the serotype they use infects medium spiny striatal cells efficiently, but she was uncertain about its ability to infect other cells, such as cortical cells which have been implicated as major players in HD pathology. David Housman added it will also be interesting to know the efficiency of glial versus neuronal infection. To help distinguish the effects of RNAi on different cell types, C. Johnson suggested using cell type-specific promoters and William Yang offered to provide mice developed by Nat Heintz which have differentially-labelled cell types. Regarding AAV’s safety, Davidson noted that the effects of neurosurgical application of AAV have been well studied in non-human primates and humans. Non-human primates have been followed for 6 years with no detectable adverse consequences.

Non-viral delivery systems were also discussed. Kuhn noted that researchers at Novartis have used pumps to deliver formulated RNA oligonucleotides in mice using continuous perfusion for two weeks. Diffusion of the oligonucleotides into the brain is poor when pumps are placed in the ventricles, but when placed in the striatum, diffusion is sufficient to cover the entire striatum, said Davidson. She also noted that her collaborator has succeeded in covering the striatum and putamen using convection-enhanced delivery of lipid-based RNA formulations. Anne Young asked whether simply injecting oligonucleotides into the bloodstream every couple of weeks would be feasible. In addition to minimizing invasivity, this option could target all neurons of the brain, a desirable consequence given growing evidence of mutant huntingtin’s widespread effects. Davidson is considering panning for viruses that cross the blood-brain barrier which would be useful for this application, but she also noted that intravenous administration might require prohibitively large amounts of oligonucleotides. Nasal sprays are yet another option, as pointed out by Zaven Khachaturian. However, Davidson said her group’s attempts at using them have been unsuccessful so far.

Elena Cattaneo wondered about the persistence of RNAi effects. Davidson noted that viral delivery is expected to result in long-lived effects—her group has observed effects that last several months, and she is planning to monitor them a year after injection, and beyond. In non-viral systems, oligonucleotides persist for about 2 weeks, according to studies from other groups.

But as noted by Davidson, chemical modifications to enhance stability might yield longer lifetimes.

Addressing an issue specific to allelic interventions, Davidson pointed out the need for more candidate polymorphisms. Housman noted that the most useful SNPs are those present at high frequencies, and these have probably already been identified (such as the exon 58 SNP). However, he also pointed out that focusing searches on the UTRs, particularly the 3' UTR, might be fruitful.

Steven Finkbeiner wondered how many patients are expected to benefit from allele-specific therapies. Housman pointed out that the exon 58 SNP is present in approximately 40% of the HD population. He added that even polymorphisms with significantly lower frequencies are of therapeutic interest and likely to be accepted by the FDA because they are markers that can be reliably identified, and treatments could be restricted to only those who will benefit from them. As noted by Al LaSpada, it will also be important to assess, using HD models, at what stages of disease patients are likely to benefit from either allelic or non-allelic RNAi interventions.

Action items

1. Assess whether HD mice can tolerate and benefit from reductions in both wildtype and mutant huntingtin.
2. Ensure outcome measures of animal experiments provide sufficient in-depth information to allow informed decision-making for human testing.
3. Search for additional HD-associated polymorphisms.
4. Define which cell types are the best targets for RNAi intervention and ensure the delivery system is appropriately matched.
5. Pan for viruses that cross the blood-brain barrier.
6. Explore non-viral delivery options in greater depth.
7. Examine the persistence of RNAi effects and their implications for therapeutic development.
8. Test RNAi effects at different stages of disease.

General approaches applicable to various neurodegenerative disorders

Targeting the primary source of HD with approaches such as RNAi is appealing because of its directness and specificity. However, there are also advantages to tapping into more general cellular protective pathways. Harnessing the endogenous abilities that cells, particularly neurons, have evolved to protect themselves from environmental and internal insults could yield valuable therapeutic alternatives with broad applicability to neurodegenerative disorders. A major advantage to this approach is that the knowledge and experience obtained from studies of one disease can benefit others. Furthermore, studying general protective pathways can provide handles for the subsequent identification of more specific targets for particular diseases.

Indeed, a new venture that seeks to integrate resources, information and ideas relevant to various neurodegenerative disorders is currently being set up, as described by Khachaturian, a leader of the initiative. Among its planned activities, the project will bring together interested parties from the clinic, academia, industry, and government in brainstorming workshops to identify common themes and distinguishing features of neurodegenerative disorders. The

workshops are expected to help recognize important knowledge gaps, determine common resource needs and therapeutic opportunities, as well as propose new research directions. In-depth workshops focusing on specialized topics will be organized, as well as more general workshops that survey big picture issues. A systems approach will be adopted in which information at many levels, ranging from molecular to clinical, is integrated. The initiative will also generate materials for the general public, as well as create documents and presentations to raise legislative and philanthropic awareness. The project is currently being funded by philanthropists in Las Vegas, and participants include HDF members Young, Nancy Wexler and C. Johnson.

General approaches: Targeting toxic protein structures

Current status

Some researchers are probing the structure of mutant huntingtin and its aggregate forms in search of therapeutic candidates. Although the approach is closely linked to HD's primary defect, it shares surprisingly key commonalities with other degenerative disorders. Like HD, Alzheimer's disease (AD), Parkinson's disease (PD), type II diabetes, and sporadic inclusion body myositis, for example, are all characterized by the accumulation of insoluble, misfolded proteins. Furthermore, a wide variety of these proteins adopt common soluble, oligomeric configurations that may mediate similar mechanisms of toxicity.

Ron Wetzel explained that, as revealed by circular dichroism and NMR spectroscopy, polyglutamine is a classic random coil. It is "intrinsically disordered". That is, in the presence of other proteins it might adopt a particular configuration but, on its own, it does not fold into a single, well-defined structure. Greg Lemke added that, in a manner consistent with this lack of structure, the least conserved sequence in polyglutamine proteins is the length of the polyglutamine stretch.

When polyglutamines exceed a certain length, they cause their host proteins to aggregate. Long polyglutamine chains tend to aggregate. In addition, they sometimes cause conformational alterations in their host proteins which, in turn, lead to aggregation. For example, Wetzel noted that polyglutamine expansions in ataxin 3 shift the protein's folding equilibrium to an unfolded state which is more aggregate-prone.

Wetzel described various aggregate forms with distinct characteristics. Microaggregates, not directly visible by light microscopy, and inclusions, visible by light microscopy, have been described in cells. *In vitro*, oligomers, protofibrils and amyloid fibrils have been described, in order of increasing size. Wetzel clarified that the different-sized aggregates do not comprise a growth series—oligomers do not necessarily become protofibrils, nor do protofibrils necessarily become amyloid fibrils. He also emphasized that there is no one-to-one correspondence between the *in vivo* and *in vitro* categories. For example, inclusions can be made of either long, thick amyloid fibrils, or of small, spherical oligomers. These descriptions apply to aggregates formed by either polyglutamines or A β peptides, although polyglutamine protofibrils are only observed in the presence of compounds that stabilize them.

In addition to differing in size and shape, the various aggregate forms differ in their secondary structures and behaviors. Oligomers and protofibrils have α -helices and low β -sheet content, whereas amyloid fibrils have high β -sheet content. In addition, amyloid fibrils grow well by monomer addition, while oligomers and protofibrils do not. This particular feature can be studied, noted Wetzel, using an assay developed by Alex Osmand in which brain slices or

cultured cells are incubated with biotinylated poly-glutamine peptides at physiological concentrations to detect sites that can actively recruit monomeric poly-glutamine molecules. In PC12 cells expressing a GFP-labeled polyglutamine protein, large non-recruiting inclusions, and small recruiting foci are observed.

Challenges and open questions

Participants posed several questions about the characteristics of different aggregate forms. For example, Susan Lindquist asked about the selectivity of aggregate recruitment in mammalian cells. She noted that, in yeast, some protein aggregates are very selective, recruiting only monomers of their own kind, whereas other aggregates sequester many different types of proteins. Wetzel proposed using Osmand's assay, with different biotinylated monomers to examine this question.

Participants also wondered about the reversibility of aggregate formation. Wetzel noted that most inclusions grow, but some disappear spontaneously. In addition, studies using Ai Yamamoto's inducible mouse model of HD indicate that huntingtin aggregates can be cleared if huntingtin expression is turned off. It appears that autophagy and chaperone-mediated disaggregation are capable of clearing at least some types of aggregates (see following sections). Wetzel noted that pulse-chase experiments might provide a means to examine in greater depth the reversibility of aggregate formation.

Although several studies indicate that the presence of visible aggregates does not correlate well with HD pathogenesis, Wetzel emphasized that it is too early to rule out aggregates as therapeutic targets. Much remains unknown about their different forms and behaviors. Furthermore, there is strong evidence indicating that oligomers may be important mediators of toxicity. Wetzel pointed out that aggregates adopt many different forms but, therapeutically, it is only necessary to focus on blocking pathways that lead to the formation of toxic aggregates. He added that a drug need not act stoichiometrically—it could target the formation of nuclear cores, for example. Lindquist noted that Vernon Ingram's group has identified a compound that dissolves A β amyloid at equimolar concentrations, apparently by intercalating into aggregate fibers, and at lower concentrations, prevents the formation of amyloid. The compound also acts on prion aggregates.

Participants also discussed the effects of trehalose, a disaccharide reported to reduce polyglutamine aggregation. Tanaka and colleagues found that various disaccharides reduce polyglutamine aggregates and increase survival in a cellular model of HD (Nature Med, 2004). In addition, they reported that oral administration of trehalose, the most effective of the disaccharides tested, decreases polyglutamine aggregates in cerebrum and liver, improves motor dysfunction, and extends lifespan in a transgenic mouse model of HD.

Lindquist explained that trehalose affects the hydration properties of proteins, often stabilizing their folded conformations. She hypothesized that, in Tanaka's study, trehalose is mediating general folding effects that tend to reduce aggregation. Indeed, her group has observed trehalose anti-aggregation effects working with α -synuclein. She noted, however, that the Tanaka study is limited because the authors did not assess trehalose's ability to cross the blood-brain barrier and they dissolved the compound in water, a solvent in which trehalose is fairly unstable. Wetzel added that it is not clear how trehalose mediates its effects *in vivo* because, *in vitro*, enormous concentrations are required to observe its stabilizing effects. Lindquist agreed that very high concentrations (1M in yeast) are needed to see maximal effects, but the compound is well tolerated. Humans have enzymes that break down trehalose, but perhaps it is possible to

overwhelm these enzymes and achieve high concentrations without inducing toxicity. She added that confirming Tanaka's study and testing trehalose-related compounds would be worth pursuing.

Participants also briefly discussed cystamine, a compound previously thought to ameliorate HD pathology by reducing aggregate formation. Cystamine has been reported to extend survival, improve body weight and motor performance, and delay neuropathology in HD mice (Dedeoglu et al, 2002). In addition, Bob Hughes noted that combining cystamine and mithramycin—an antibiotic that regulates transcription, possibly through the downregulation of histone methylation—has yielded particularly promising results. It was previously hypothesized that, through its actions as a transglutaminase inhibitor, cystamine could reduce HD aggregate formation. As noted by C. Johnson, however, cystamine is beneficial even when transglutaminase is knocked out, indicating that it is acting through an alternate pathway. Because cystamine has many effects, including inhibiting caspase activity and increasing antioxidant levels, its mechanism of action in HD remains unclear.

Searching for anti-aggregation agents systematically, Wetzel and colleagues are screening compound libraries monitoring the recruitment of fluorescently labelled monomers. So far, they have identified several compounds that inhibit monomer recruitment. However, some of these molecules increase, rather than decrease, aggregate size, as assessed by light scattering. The compounds appear to be promoting protofibril growth in a manner that does not involve monomer addition, explained Wetzel. Although the compounds might be useful for producing non-toxic aggregates, he noted that for therapeutic purposes, it might be best to use a less specific assay. Paolo Paganetti and Chesselet added that assessing the effects of these compounds in cells, such as PC12s, might be worthwhile.

Action items

1. Use Osmand's assay to examine the recruitment selectivity of different polyglutamine aggregates.
2. Conduct pulse-chase experiments to explore the reversibility of aggregate formation.
3. Confirm trehalose results and test related compounds.
4. Follow up on Wetzel's drug screening results.
5. Develop new aggregation assays that are not limited to tracking monomer recruitment.

General approaches: Modulating heat shock protein networks

Current status

An alternative to searching for compounds that inhibit aggregation is to find ways of modulating cells' endogenous abilities to deal with aberrant proteins. Several studies have indicated that the regulation of components of the cellular quality control machinery, specifically heat shock proteins (Hsp), may be a promising therapeutic target for several protein conformational diseases. Richard Morimoto presented a brief overview of this area of research, highlighting issues of specific relevance to HD.

Morimoto explained that many of the proteins encoded by the heat shock gene superfamily act as molecular chaperones involved in the reversible transformations of unfolded protein chains to intermediate structures and, of intermediate structures to fully folded, mature proteins. In addition, chaperones can regulate the self-association of unstable intermediate structures which lead to the formation of oligomers and inclusions.

One of the distinctive characteristics of molecular chaperones is that they function in networks. The heat shock response (HSR) involves a multistep pathway with both positive and negative regulators, where the heat shock transcription factor 1 (HSF1) acts as the master regulator. In response to a variety of stresses, HSF1 trimerizes and binds with high affinity to the heat shock elements (HSEs)—inverted repeats of the sequence nGAAn located in the promoter regions of Hsp genes (several Hsp genes are also transcribed constitutively at low levels which vary between cell types). A negative feedback loop, involving the downregulation of HSF1 by Hsp70 and Hsp90, helps limit the response.

In addition to HSEs, Hsp gene promoters contain binding sites for many other stress-associated transcription factors, including Nrf2 and *daf-16* (see sections below). Thus, Hsps act as integrators of stress signals. Furthermore, as pointed out by Lindquist, Hsp levels are regulated not only at the transcriptional level, but at all levels of gene expression, including mRNA stability and translation.

Another distinguishing feature of molecular chaperones is that their activities are highly modulated, noted Morimoto. For example, there are dozens of co-chaperones that regulate the Hsp70 proteins—central components of the network of chaperones that assist protein folding in several subcellular domains. Hsp70 switches between its low-affinity ATP bound state and its high affinity ADP bound state as it goes through its substrate binding and release cycle. This ATPase cycle is controlled by co-chaperones of the family of J-domain proteins, which target Hsp70s to their substrates. In humans, 35 J-domain proteins have been described. In addition, nucleotide exchange factors and ADP/ATP ratios determine the lifetime of the Hsp70-substrate complex, and additional co-chaperones fine-tune the chaperone cycle. Some regulators enhance Hsp70 activity, while others inhibit it. For example, the Hsp70-interacting protein Hip stimulates Hsp70, while Bag-1 inhibits it. Also, the Hsp70 cycle is coupled to the action of other chaperones, including Hsp90 and Hsp100.

Several lines of evidence indicate that manipulating the HSR could be beneficial for HD. A recent study from David Rubinsztein's group, for example, indicates that overexpression of yeast Hsp104 reduces polyglutamine aggregation and prolongs survival of a transgenic mouse model of HD. In addition, Morimoto's group has found that overexpression of HSF1 in *C. elegans* suppresses polyglutamine protein aggregation, whereas reduction of HSF1 by RNAi potentiates aggregation. Of particular interest, Morimoto noted that a potent activator of HSF1 was recently identified by the NINDS Neurodegeneration Drug Screening Consortium. The compound, known as celastrol, is a triterpenoid derived from the *Celastraceae* family of plants, and an active component from Chinese herbal medicine. Anticipating its potential therapeutic value, a large number of celastrol derivatives have been synthesized. Lindquist added that her group has identified an active component from Indian herbal medicine which induces HSF1 and appears to ameliorate mutant huntingtin toxicity in PC12 cells.

Furthermore, Morimoto noted that several compounds that target the HSR are already in clinical trials. For example, 17AAG—an analogue of geldanamycin which binds to Hsp90, affects the stability of short-lived proteins, and causes derepression of HSF1 and consequent activation of the HSR—is now in phase III clinical trials for cancer. Of relevance to HD, geldanamycin has been reported to inhibit the formation of huntingtin aggregates in cell cultures and brain slices. However, as noted by Gill Bates, 17AAG does not cross the blood-brain barrier. Morimoto added that Hsp90 is one of the 5 most abundant proteins in cells and Hsp90 inhibitors

are likely to have multiple effects. To address this issue, drugs are being administered in pulses, rather than given chronically.

Proteasome inhibitors are another set of drugs that activate the HSR and are now in phase III clinical trials. To limit secondary effects, they are being given at low concentrations to only partially activate the HSR. In addition, non-steroidal anti-inflammatory drugs (NSAIDs) can act as co-inducers of the HSR. These compounds partially activate components of the HSR and, in the presence of a secondary stress signal, can fully induce Hsp70 expression.

Challenges and open questions

Although encouraging, it is still unclear how these efforts and observations may translate into therapeutic benefits for HD. Morimoto noted that HD cells appear to have a low-level activation of the HSR, but the reasons for this are unknown. He added that the stress associated with HD is chronic, a condition which affects cells very differently than most commonly studied acute, stressful stimuli.

In addition, modulating the HSR therapeutically has several associated challenges. Although the multiple endogenous positive and negative regulators of the HSR provide many potential targets for small molecule intervention, the complexity of the network makes the consequences of particular manipulations difficult to predict. For example, Morimoto's group has observed that in 12 clonal cell lines co-expressing a polyglutamine chain with 40 glutamines and labelled Hsps, only a fraction of the cells activate the HSR and have correspondingly fewer aggregates. Morimoto is uncertain about the reason for this heterogeneity, but he suspects that small perturbations in cellular conditions can translate into dramatic HSR effects. To help clarify the effects of modulating the HSR in HD, Lindquist's group is setting up crosses between HSR mutants, and R6/2 and Borchelt HD mice.

Another challenge in the field, as noted by Lindquist, is the tight cellular regulation of HSR components which makes it difficult to artificially modulate their levels. For example, when one tries to overexpress Hsps experimentally, they aggregate and become inactive. Not only does this prevent Hsp activity from being increased, the aggregation can be toxic. Lindquist noted that her group has resorted to modifying Hsp 3' UTRs to increase their levels. But even manipulations of this kind have their shortcomings. As previously mentioned, Hsp concentrations are regulated at many different levels (e.g., transcriptional, mRNA stability, translational), so cells may compensate for alterations that target a single regulatory level.

A lack of knowledge of some key features of the HSR is also problematic. Morimoto noted that the concentrations of 150 chaperones in different cells are unknown. To resolve this question, Lindquist pointed out, proteomics experiments will be needed because of the major role post-transcriptional regulation plays in determining Hsp levels.

Targeting HSR components that are specific to neurons, or even to particular neuronal types, could yield more powerful and more specific therapeutic candidates. Yet, as noted by Lemke, not much is known about cell type-specific Hsps and Hsp regulators. So far, promising results have emerged from generally boosting HSF1 activity. Indeed, beyond its effects on polyglutamine aggregation, Morimoto pointed out that overexpressing HSF1 in worms increases longevity. Nevertheless, associated side-effects could be problematic. As noted by Lindquist, many oncogenic proteins require Hsps for their activity and most tumors express high Hsp levels. In general, increasing Hsp activity appears to be beneficial for neurodegeneration, but detrimental for cancer.

A future alternative to boosting the HSR, suggested Morimoto, might be to harness chaperones' abilities to recognize misfolded proteins and target them to proteasomes. As this mechanism is understood in greater depth, in particular the connection between protein folding and routing, it may generate new therapeutic avenues.

Action items

1. Follow up on the therapeutic potential of celastrol, celastrol derivatives, and Lindquist's HSF1-inducing compound.
2. Cross HD mice (R6/2 and Borchelt) with HSR mutants.
3. Examine options for manipulating chaperone-mediated protein folding and routing systems.

General approaches: Boosting cellular clearance mechanisms

Current status

An alternative to manipulating the HSR to reduce the formation of toxic mutant huntingtin species is to induce cellular clearance mechanisms to eliminate them. As explained by Ana Maria Cuervo, there are various protein clearance systems in the cell. All cellular proteins, not only misfolded ones, are turned over by either the ubiquitin proteasome system (UPS) or lysosomes. Lysosomes, of which there are four different types, mediate heterophagy—the degradation of proteins internalized through endocytosis—and autophagy, the degradation of intracellular components. There are three recognized forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). During macroautophagy, double-membrane sequestering vesicles, or autophagosomes, fuse with lysosomes. During microautophagy, cytosolic components are directly transferred into the lysosome by invagination of the lysosomal membrane. And during CMA, specific cytosolic proteins are translocated across the lysosomal membrane via the lamp2A receptor which recognizes the CMA consensus motif KFERQ. Soluble, monomeric proteins are usually degraded by the proteasome or, in some cases, via CMA. Protein complexes, aggregates, and entire cellular organelles, on the other hand, are cleared by micro- and, most commonly, macroautophagy.

Challenges and open questions

As pointed out by Cuervo, a major unresolved question is how cells normally clear wildtype and mutant huntingtin. The UPS appears to contribute to huntingtin clearance but, in disease, it might also be the source of toxic fragments that nucleate aggregates which, in turn, disrupt UPS function.

CMA may also be involved in huntingtin clearance, but only under certain conditions. Cuervo and her colleagues recently reported that wild-type α -synuclein is degraded by CMA, and the pathogenic A53T and A30P α -synuclein mutants appear to act as uptake blockers, inhibiting their own degradation and that of other substrates (Cuervo et al, 2004). Observing that huntingtin has 5 CMA motifs, Cuervo tested if it too is degraded by this pathway. The group found that, despite its motifs, full-length, wildtype huntingtin is not degraded by CMA. However, huntingtin that has been post-translationally modified might be a substrate for CMA. In particular, Cuervo plans to test whether modifications in a sequence found in exon 1 might mediate CMA through the creation of a consensus-like motif.

Moreover, several studies have implicated macroautophagy in mutant huntingtin clearance. One of the first indications was provided by Marion DiFiglia and colleagues who observed huntingtin-labeled vacuoles with ultrastructural features of macroautophagosomes in HD neurons (Kegel et al, 2000). Subsequently, David Rubinsztein and colleagues have observed an accumulation of tagged mutant exon 1 when cells are treated with inhibitors of various stages of the macroautophagy pathway. Moreover, rapamycin, which activates macroautophagy, enhances the clearance of the mutant protein, protects against neurodegeneration in a fly model of HD, and improves behavioral symptoms in N171 mice (Ravikumar et al, 2004).

The relative contributions of these different clearance pathways and how they change during disease progression, however, is unknown. As noted by Cuervo, the same protein can be degraded by multiple pathways. Also, there is cross-talk between clearance pathways, such that blocking one, can result in the upregulation of another. Furthermore, autophagy regulation varies between cell types. In HD, the question of pathway contribution is even more complicated because there are many huntingtin fragment species, as well as many types of huntingtin aggregates which probably have different fates.

Another complication is the complex, still ill-defined interactions between mutant huntingtin and the various clearance systems. As mentioned above, the UPS might generate toxic mutant huntingtin fragments which then cause impairment of UPS function. Mutant huntingtin may also interfere with autophagy—the slow rate of huntingtin clearance and the persistence of autophagosome structures are consistent with this possibility. In addition, the disruption of neurite trafficking caused by huntingtin aggregates could affect autophagy. Cuervo noted that autophagosomes are observed throughout neurites and macroautophagy may occur at a low frequency within these structures. In addition, as pointed out by Marina Chicurel, wildtype huntingtin may itself play a role in autophagy which is disrupted when the protein is mutated.

Genetics may help address some of these questions, but there are associated challenges. As noted by Cuervo, over-expressing autophagy (Atg) proteins is problematic because they require post-translational modifications to function properly. In addition, mammals appear to have proteins that overlap in their functions with Atgs, but these proteins have not been well characterized. Mice that overexpress the Atg protein beclin-1 are available, but beclin-1 has been implicated in other cellular processes, such as apoptosis.

Despite these uncertainties, several studies suggest that stimulating autophagy could have therapeutic potential. As previously mentioned, Rubinsztein found that rapamycin—which activates autophagy by inhibiting the regulatory protein kinase, target of rapamycin (TOR)—protects against neurodegeneration in a fly model of HD, and improves behavioral symptoms in HD mice. In addition, Cuervo noted that Rubinsztein's group has found that lithium, acting through a mTOR-independent pathway, enhances the clearance of mutant huntingtin.

The lithium results are of particular interest because mTOR is limited in its potential as a therapeutic target. In addition to autophagy, mTOR regulates fundamental cellular processes such as cell growth and cell cycle progression. Also, rapamycin's effects on autophagy are transient. Moreover, mTOR is inhibited strongly by mutant huntingtin. Thus, trying to inhibit mTOR further with rapamycin may be only mildly effective. Cuervo noted that it may be worth examining the clinical records of HD patients who have been on lithium for mood disorders.

Hughes noted that inhibition of mTOR is associated with increased lifespan, and wondered whether caloric restriction and low nitrogen diets, which increase lifespan, are capable of downregulating mTOR. Cuervo said that, indeed, one of the classic ways of activating autophagy is through starvation and, at least in yeast, amino acid restriction, in particular, is

known to induce autophagy. Interestingly, as noted by Leslie Thompson, R6/2 mice appear to benefit from caloric restriction. However, HD patients are advised to eat large amounts of food, particularly high-fat foods, because increased weight seems to slow disease progression. Whether and how these high caloric diets affect autophagy is unknown.

Some potentially undesirable consequences of generally activating macroautophagy were also discussed. As noted by Cuervo, macroautophagy can act as a mediator of cell death under some conditions. In addition, the regulation of macroautophagy is not specific to particular substrates. Thus, upregulating it could affect any cellular component cleared through this pathway. Indeed, as pointed out by Chicurel, in α_1 -antitrypsin (α_1 -AT) deficiency, autophagy is induced by endoplasmic reticulum retention of α_1 -ATZ which affects mitochondria, resulting in mitochondrial autophagy and injury. As noted by Cuervo, it is possible that the goal for effectively manipulating macroautophagy in neurodegenerative diseases is to maintain a certain level of autophagy, rather than increase it dramatically beyond normal levels. In addition, Cuervo suggested that identifying the components or features of aggregates that are recognized by the autophagic machinery might suggest new strategies to specifically boost aggregate clearance.

Action items

1. Assess whether post-translational modifications of full-length huntingtin result in CMA-mediated clearance.
2. Use genetics to confirm and extend knowledge about the relationships between autophagy and HD.
3. Extend studies to investigate lithium's potential as an activator of autophagy—examine clinical records of HD patients who have taken lithium for mood disorders.
4. Identify aggregate components or features recognized by the autophagic machinery.

General approaches: Regulating the Nrf2-ARE signalling pathway

Current status

Regulating pathways involved in stress management, such as the Nrf2-ARE pathway, is another approach that may help ameliorate HD pathology. As explained by Jeffrey Johnson, nuclear factor erythroid 2-related factor 2 (Nrf2) (not to be confused with nuclear respiratory factor 2 (NRF2)) is a transcription factor that regulates the coordinate expression of genes involved in detoxification and antioxidant activities. Nrf2 is sequestered in the cytoplasm by Keap1, a small protein with 21 cysteine residues that is postulated to be the redox-sensing system of the cell. In response to oxidative or chemical stress, Keap1 releases Nrf2 allowing it to translocate into the nucleus and bind to the antioxidant responsive element (ARE) found in the promoters of antioxidant and detoxification genes, including those involved in maintaining cellular levels of NADPH, ATP, and glutathione.

The pathway protects cells against insults that act through the disruption of mitochondrial function. Thus, many neurodegenerative disorders which involve mitochondrial disruptions associated with cell death—including HD, AD, PD, and amyotrophic lateral sclerosis (ALS)—could potentially benefit from the activation of Nrf2. Nrf2 does not directly switch off the apoptotic pathway, but it enhances cells' abilities to cope with and eliminate apoptotic stimuli such as reactive oxygen species. It can be considered a master switch of phase II metabolic enzymes, the enzymes that handle the products of xenobiotic compounds generated by phase I

enzymes. Phase II enzymes, such as glutathione-S-transferase and the N-acetyl transferases, are responsible for the inactivation of xenobiotics prior to excretion.

To examine Nrf2's role in protecting neurons from stressful conditions, J. Johnson's team analyzed how Nrf2-deficient cells and Nrf2 knockout mice respond to malonate and 3-nitropropionic acid (3NP)—mitochondrial complex II inhibitors which cause striatal damage similar to that observed in HD (Calkins et al, PNAS, 2005). The data revealed that both systems were significantly more vulnerable to the chemical toxins than wildtype controls. In addition, the team observed that, in wildtype cells, the toxins increase ARE-regulated transcription mediated by astrocytes. Moreover, transplantation of Nrf2-overexpressing astrocytes into mice striata results in complete protection from malonate toxicity. According to J. Johnson's calculations, between 500 and 700 astrocytes are sufficient to protect the entire striatum. *In vitro* studies indicate that a single astrocyte can protect approximately 700 cortical neurons.

To extend these findings, J. Johnson is now working with various animal models of neurodegenerative diseases, including G93 transgenic mice deficient in Cu,Zn superoxide dismutase, and the R6/2 and Borchelt mouse models of HD (see below).

Challenges and open questions

The mechanisms by which Nrf2 protects neurons in J. Johnson's experiments remain uncertain. To identify the protective compound(s) secreted by astrocytes, J. Johnson is analyzing astrocyte supernatants using a proteomic approach that includes liquid chromatography/mass spectrometry, as well as mass spectrometric analysis of two-dimensional gels. As noted by Yang, the key role of astrocytes is consistent with recent findings from Xiao-Jiang Li's group who reported that, in a neuron–glia coculture system, wild-type glial cells protect neurons against mutant huntingtin-mediated neurotoxicity, whereas glial cells expressing mutant huntingtin increase neuronal vulnerability (Shin et al, JCB, 2005). One contributing factor to these results might be that the Nrf2 pathway is dysfunctional in HD astrocytes. Interestingly, Michael Levine has found that exercise, which normally induces Nrf2 activation, is detrimental to R6/2 mice.

Chicurel wondered whether neurons, in addition to astrocytes, activate the Nrf2 pathway. J. Johnson noted that the pathway is turned off during neuronal differentiation. His group is currently trying to identify the factors that trigger the switch-off.

Cuervo suggested extending J. Johnson's observations by incubating R6/2 and wildtype neurons with supernatants from either R6/2 or wildtype astrocyte cultures. J. Johnson agreed, and added that he also plans to test astrocytes deficient in glutathione production to assess whether the protection they confer is mediated by glutathione release. In addition, he is transplanting primary astrocytes over-expressing Nrf2 into the striata of R6/2 and Borchelt mice. He expects to obtain data from the Borchelt mice in 6 months, and from the R6/2 mice by the end of the year.

As participants discussed possibilities for upregulating Nrf2 signaling to treat HD, Chesselet noted that she and her collaborators have completely reversed some HD symptoms and significantly reduced neuropil aggregates using curcumin, an activator of Nrf2. However, she noted that curcumin has multiple effects in addition to stimulating the Nrf2 pathway. Also, curcumin does not cross the blood-brain barrier efficiently, so the observed effects are probably indirect, perhaps mediated by a curcumin metabolite.

As noted by J. Johnson, many compounds induce the Nrf2 pathway. According to a recent review, there are at least eight distinct chemical classes of monofunctional inducers—compounds that induce ARE-driven gene expression without significantly enhancing the activity of phase I enzymes (van Muiswinkel and Kuiperij, 2005). Most are considered to be fairly safe,

and some are found in common foods such as broccoli, green tea, and curry. Several ongoing efforts are directed at identifying Nrf2 inducers in various dietary products. Interestingly, Thompson noted that two therapeutic candidates for HD, the histone deacetylase inhibitor SAHA and the transglutaminase inhibitor cystamine, upregulate glutathione. Whether they do so through Nrf2 activation, however, is unknown.

Casting a wider net, J. Johnson's team has developed a cell-based system to conduct high throughput screens to identify molecules that activate the Nrf2 pathway. So far, they have screened 1.4 million molecules. After eliminating molecules that cause oxidative stress, they have 300 hits, some of which have shared structures. The team is now generating structurally modified variants and testing them in animal reporters to assess their effects on Nrf2 activity in the brain. Only compounds that can cross the blood-brain barrier are being pursued.

A few of the hits are FDA-approved compounds, such as pioglitazone. Pioglitazone is an anti-diabetic drug that activates PPAR- γ —a nuclear hormone receptor highly expressed in adipose tissue—as well as Nrf2. Rainer Kuhn noted, however, that pioglitazone does not reach the brain efficiently. J. Johnson agreed and explained that the positive effects they have observed may be mediated by a metabolite.

Kuhn also worried about the selectivity of the compounds identified in J. Johnson's screens. To address this issue, J. Johnson is performing transcriptional profiling using Agilent and Affymetrix microarrays to characterize the compounds' effects. He added that upregulating Nrf2 therapeutically may also require some degree of cell type specificity. Keap-1 knockout mice die at around three weeks of age from massive proliferation of epithelial cells in the esophagus.

Another issue discussed by participants was the potential therapeutic range of Nrf2 activation. J. Johnson noted that, in ALS, Nrf2 activation rises steadily in muscle, reaching up to 1000-fold towards the end of the disease. Hughes asked whether similar measurements have been obtained for HD brains. According to J. Johnson, activation is not observed in Borchelt mice, but human tests remain to be done. Lemke stressed the importance of these measurements, noting that, if Nrf2 is already strongly activated in HD, attempting to boost it higher pharmacologically may not be effective. The measurements may also be helpful in determining whether Nrf2 activation could serve as a biomarker for HD progression, noted Chesselet.

Another consideration, pointed out by Paganetti, is that potentiating phase II enzymes, which inactivate xenobiotic compounds, may be problematic in HD patients who often take many medications. However, if Nrf2 inducers ultimately prove effective in treating HD, patients may not require many additional medications.

Action items

1. Assess the effects of transplanting primary astrocytes over-expressing Nrf2 into the striata of R6/2 and Borchelt mice.
2. Use proteomic approaches to identify neuroprotective species secreted by astrocytes.
3. Identify factors that induce neurons to switch off Nrf2 signaling.
4. Examine the effects of HD and wildtype astrocytes on HD and wildtype neurons *in vitro*.
5. Test whether astrocytes deficient in glutathione secretion are neuroprotective.
6. Follow up on Chesselet's curcumin findings.
7. Continue analysis of hits identified by J. Johnson's screening assays.
8. Assess levels of Nrf2 activation in HD brains.
9. Evaluate effects of diet supplementation with Nrf2 inducers in chronic models of HD.

10. Learn about relevant industry efforts (J. Johnson noted that Merck seems to be pursuing Nrf2 research.)

General approaches: Altering histone deacetylase activity

Current status

Regulating histone deacetylases (HDACs) emerged as another important therapeutic candidate. Histone acetylation helps make tightly packed chromatin accessible to transcription factors, thereby increasing gene transcription. HDAC inhibitors have been shown in several systems to ameliorate HD pathology. However, activation of certain HDACs, in particular the Sir2 sirtuins, appears to ameliorate HD pathology. It is likely that differences in substrate specificity underlie these apparently contradictory results. Participants discussed both sets of observations and their potential for future therapeutic development.

Several lines of evidence suggest that gene expression is altered in HD, and abnormalities in the acetylation and deacetylation of histones may contribute to this alteration. For example, as noted by Thompson, the levels of acetylated histones H3 and H4 are decreased in animal models of HD. In addition, studies in yeast, *Drosophila*, and mice, indicate that HDAC inhibitors mitigate the toxic effects of polyglutamines. Based on these observations, Gill Bates noted that her group has been testing the effects of several HDAC inhibitors on HD mice. So far, only suberoylanilide hydroxamic acid (SAHA) has proven efficacious. Administered at 100 mg/kg, SAHA improves rotarod performance in R6/2 mice dramatically, and reverses 50% of HD-associated alterations in the cerebellar transcriptome. In contrast, several other inhibitors reported to benefit HD, such as sodium butyrate, have been ineffective. As noted by Bates, this may be explained by her system's stringency and the compounds' insufficient potency and/or inability to efficiently cross the blood-brain barrier.

Summarizing recent data supporting the benefits of enhancing, rather than inhibiting, HDAC activity, Christian Neri described how activating the Sir2 sirtuins, a group of NAD-dependent HDACs, appears to ameliorate HD toxicity. Sir2 sirtuins are best known for regulating lifespan in yeast and worms: deleting them decreases replicative lifespan, while over-expressing them increases lifespan. In mammals, Sir2 regulates the FOXO transcription factors. FOXO proteins act as stress sensors, and control the expression of genes that regulate apoptosis, and pathways involved in resistance to oxidative and chemical stress. Approximately 600 genes have been identified as downstream effectors of FOXO. Neri believes that, acting through FOXO, Sir2 can turn on two sets of distinct, but partially overlapping, genes: one that regulates longevity, and another that regulates neuronal survival.

Based on previous studies in *C. elegans* suggesting that regulators of *daf-16*, a member of the FOXO family, might be importantly involved in cell protection against polyglutamine toxicity, Neri and colleagues recently tested whether increased dosage of *sir-2.1*, or treatment with the sirtuin activator resveratrol, could ameliorate polyglutamine toxicity. The team reported that, indeed, both these manipulations, acting through *daf-16*, specifically rescue neuronal dysfunction induced by expanded polyglutamines, as assessed by tail mechanosensitivity, and reduce the number of dystrophic cell processes (Parker et al, Nat Genet., 2005). In addition, the researchers showed that resveratrol can rescue cultured neurons derived from HdhQ111 knock-in mice from cell death.

Challenges and open questions

Participants noted a number of unresolved issues regarding the development of HDAC inhibitors as therapeutic candidates for HD. As summarized by C. Johnson, these compounds appear to be worth pursuing, but their path to the clinic is proving less straightforward than originally expected. Bates noted that, despite its robust effects, SAHA has several limitations. It has toxic side-effects, causing weight loss in mice. Dosing experiments may help identify a window that minimizes these effects, but identifying other effective inhibitors will also be important. In addition, Bates noted the importance of assessing different inhibitors' concentrations in the brain.

Several participants noted that identifying the HD relevant targets that SAHA and other HDAC inhibitors act upon is a top priority. As pointed out by Thompson, the specific deacetylases and their relevant substrates need to be pinpointed. Indeed, it is still unclear whether histones are, in fact, the affected substrates mediating HDAC inhibitors' beneficial effects. Kuhn agreed, noting that it is very difficult to optimize candidate therapies if the molecular goal of the optimization is unknown. He added that making an HDAC inhibitor more potent, without tailoring its potency to the set of genes of interest, can result in greater toxicity, as has occurred with HDAC inhibitors developed for cancer.

Bates noted her group is very interested in beginning to refine HDAC targets using a genetic approach. She noted that HDAC I and V knockouts are viable and can be crossed with R6/2 mice. In addition, Bates is interested in testing additional pharmacological inhibitors, including class III inhibitors which she has yet to test in mice. Participants also suggested using RNAi in embryonic stem (ES) cells to generate mice with different types of HDACs knocked down. C. Johnson added that the approach could be directly applied to R6/2 ES cells. In flies, noted Thompson, decreasing HDAC classes I and III, but not II, is effective in alleviating HD toxicity. Interestingly, the simultaneous reduction in classes I and III results in a synergistic effect.

Despite the above mentioned uncertainties, one HDAC inhibitor, phenyl butyrate, is already in clinical trials. Studies in N171 mice showed that phenyl butyrate significantly extends survival and attenuates both gross brain and neuronal atrophy (Gardian et al, 2005). Young noted that this compound is currently being tested for safety and tolerability.

Participants also discussed open questions regarding the Sir2 sirtuins. For example, they stressed the importance of determining whether sirtuin regulation can ameliorate HD pathology *in vivo*. To address this issue, Neri plans to cross sirtuin over-expressing mice with various HD mouse models. As a complement to these experiments, C. Johnson suggested crossing sirtuin knockout mice from Lindquist's lab with HD mice.

Another issue discussed was resveratrol's limitations as a Sir2 activator. As noted by Kuhn, the compound is unstable. Also, Neri pointed out it does not cross the blood-brain barrier efficiently. Furthermore, there is some indication that resveratrol may affect Sir2 functions that are independent of FOXO, in particular the regulation of endoplasmic reticulum stress genes. Moreover, resveratrol blocks the aryl hydrocarbon receptor (AHR)—a master switch of phase I enzymes—and promotes intracellular degradation of amyloid β peptides via a mechanism that involves NF- κ B signaling and the proteasome. Bates noted she has observed positive effects in HD mice using resveratrol, but she suspects they are not mediated by Sir2. An additional complication is that Sir2 may have different effects in dividing versus non-dividing cells. As

noted by Bates, a recent study indicates that, in yeast, the effects of Sir2 on chronological life span are opposite to its effects on replicative life span (Fabrizio et al, 2005).

To bypass at least some of these complications, Neri is interested in identifying resveratrol analogs that regulate *daf-16* exclusively. Therefore, he has developed an acetylation assay, using fluorophore-labelled FOXO peptides, to search for additional regulators of Sir2. The assay has identified iso-nicotinamide and resveratrol as activators, and nicotinamide and sirtinol as inhibitors. Neri emphasized that more fluorophore-labelled compounds are needed to dissect associated biochemical pathways and identify regulatory agents.

An additional issue, noted by Kuhn, is that the upregulation of Sir2 lacks specificity as a therapeutic candidate for HD or neurodegeneration. However, Neri pointed out that the *daf-16* pathway is very well characterized so that, in addition to being itself a neuroprotective target, it can serve as a handle to discover new, potentially more specific, targets. Understanding the mechanism by which resveratrol and sirtuins mediate their protective effects is likely to provide targets that are more specific for polyglutamine toxicity. To pursue this search, Neri is using *C. elegans* genetics and cell-based assays. In particular, he is setting up RNAi screens of the downstream effectors of FOXO to identify proteins that confer polyglutamine protection.

Participants wondered whether targeting members of associated signaling pathways might also be worthwhile. For example, the insulin-like signaling pathway inhibits FOXO through phosphorylation. Interestingly, the protein kinase Akt, which is part of this pathway, has been implicated in huntingtin phosphorylation. However, Neri pointed out that inactivating insulin-like growth factor-1 is less effective and less specific for activating FOXO than over-expressing Sir2. Another pathway connection, pointed out by Morimoto, is the collaboration of heat shock factors (HSF) and *daf-16* in regulating chaperone function. In addition, J. Johnson noted that when his group screened yeast for proteins that bind *tert*-butylhydroquinone (tBHQ), a stabilizer of Nrf2, they obtained a single hit: Sir2. Although tBHQ extends lifespan in mammalian cells, he noted, the screening result is surprising because it is in contradiction with other mammalian studies. One possibility is that tBHQ binds differentially to yeast and mammalian Sir2. Indeed, Neri noted that differences between species in this pathway abound, often making comparisons difficult.

Action items

1. Identify targets of effective HDAC inhibitors:
 - a. Perform genetic crosses of HD mice with various HDAC mutants.
 - b. Knock down different HDAC types in HD ES cells using RNAi.
 - c. Identify downstream genes affected by HDAC inhibitors.
 - d. Determine whether non-histone targets are involved in effects mediated by HDAC inhibitors.
2. Optimize dosages and monitor brain levels of HDAC inhibitors.
3. Cross HD mice to sirtuin mutants.
4. Continue the search for Sir2 regulators.
5. Develop additional fluorophore-labelled reporters to examine relevant signalling pathways.
6. Use RNAi screening to identify downstream effectors of FOXO that mediate protection against polyglutamine toxicity.
7. Examine associated signaling pathways to search for additional neuroprotective targets.

General approaches: Targeting mitochondrial dysfunction

Current status

Several examples indicate a strong link between mitochondrial dysfunction and neurodegeneration. As noted by Flint Beal, many known mitochondrial disorders have an associated neurodegenerative phenotype. For example, NARP (neuropathy, ataxia, and retinitis pigmentosa), MELAS (mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes), and myoclonus epilepsy with ragged red fibres (MERRF) are all mitochondrial disorders having ataxia as a major symptom. Conversely, many neurodegenerative diseases are associated with mitochondrial alterations. For example, all of the major mutations associated with Parkinson's disease involve mitochondria.

In HD, there are also several indications of a link between neurodegeneration and mitochondrial dysfunction, noted Beal. For example, the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP) causes striatal damage similar to that observed in HD. In addition, several mitochondrial alterations have been described in HD patients, including impairments in complex II function, aconitase activity, and glucose metabolism. Also, lactate levels are increased in the basal ganglia and parts of the cortex, and phosphocreatine levels in muscle are altered.

Of particular interest, LaSpada noted his group has observed a progressive decline in body temperature in various mouse models of HD which is associated with alterations in mitochondrial numbers and function. Borchelt mice start cooling down at approximately 12 weeks of age, N171 mice show temperature disruptions as early as 8 weeks of age, and R6/2 mice experience a major drop in temperature 3 to 4 weeks prior to death. Such effects were not observed in mouse models of other neurodegenerative disorders.

LaSpada's group also observed that HD animals' brown fat, a thermogenic tissue found in rodents, looks white. This tissue is normally brown because it is heavily vascularized and its cells have high numbers of mitochondria. LaSpada found that mitochondrial function is compromised and there are less mitochondria in HD brown fat, as well as in HD cerebellum and striatum. In addition, the mRNA and protein levels of uncoupling protein 1 (UCP-1), which is critical for generating heat in brown fat by uncoupling the mitochondrial proton gradient, are low and do not respond to upregulation. Given HD's known transcriptional alterations, LaSpada's team is currently investigating whether a disruption at this level might explain the observed abnormalities. In particular they are focusing on PGC1- α , a transcriptional co-activator which is the master regulator of the cold temperature response. PGC1 α stimulates nuclear respiratory factor 1 (NRF1) to induce mitochondrial polymerase expression which is required for the replication of the mitochondrial genome. In addition, PGC1- α activates PPAR- γ which, in turn, induces the expression of UCP-1.

Challenges and open questions

As noted by Beal, the causal links between mitochondrial alterations and huntingtin toxicity remain uncertain. Although the issue is subject to debate, some evidence suggests that mitochondrial impairment is a relatively upstream event in HD. Beal noted that huntingtin associates with the mitochondrial outer membrane and its levels seem to be increased at least three-fold in HD patients. Current work using electron microscopy appears to confirm these results. Beal also noted that mutant huntingtin's deleterious effects on transcription affect the production of nuclear-encoded mitochondrial biogenesis factors. Furthermore, mutant

huntingtin's disruption of axonal transport may also interfere with normal mitochondrial function. C. Johnson added that some data suggest mutant huntingtin impairs mitochondrial fission.

To follow up on his intriguing observations, LaSpada is currently examining PGC1- α activity in various HD mouse tissues, particularly the striatum. Interestingly, as noted by Beal and LaSpada, two independently generated PGC1- α knockout mouse lines suffer from striatal degeneration. In addition, Young suggested analyzing Jim Olson's and collaborators' microarray data to assess whether mitochondrial gene expression is significantly altered as expected. Olson's data are particularly valuable because they include tissues from various HD models, as well as human tissues. LaSpada noted that Andy Strand has reported changes in the expression of mitochondrial genes in muscle, but acknowledged he needs to examine Olson's data in greater detail. In particular, he is interested in evaluating the expression of genes known to be regulated by PGC1- α .

Cuervo suggested examining whether mitochondrial turnover is affected. In aging, she noted, mitochondrial turnover is impaired. Damaged mitochondria are not cleared effectively and, consequently, the feedback mechanism that triggers mitochondrial biogenesis is not induced.

LaSpada noted he is also interested in assessing the downstream effects of altered thermoregulation. His team has noticed a correlation between thermoregulatory problems and premature death: YAC and knock-in models of HD, which have relatively long lifespans, do not have noticeable thermoregulatory alterations. Thus, LaSpada hypothesizes that these alterations may be lethal. To test this idea, he plans to examine if warming mice that do not thermoregulate normally can extend their survival.

Whether human HD patients have any abnormalities in thermoregulation is unknown. Beal said it seemed to him that HD patients don't run fevers as often as controls, but this has not been examined systematically. If humans do experience body temperature alterations, they could potentially serve as biomarkers of HD progression, suggested Wexler. Alternatively, noted Lemke, mitochondrial counts could be used as biomarkers. Wexler noted that frozen tissues and lymphoblasts from Venezuelan patients are available to evaluate this possibility. To avoid potential artefacts due to transformation of lymphoblast cell lines, LaSpada suggested obtaining fresh, peripheral blood samples.

Therapeutic directions

Compared to other candidates, some compounds that act on mitochondria are particularly far along in the HD therapeutic pipeline. As pointed out by Young, coenzyme Q10 (CoQ10) and creatine are currently in human clinical trials. CoQ10 is an essential cofactor of the mitochondrial electron transport chain, as well as an antioxidant particularly effective within mitochondria. In a 30-month randomized, placebo-controlled trial of CoQ10 (600 mg/day), remacemide, or both, neither CoQ10 nor remacemide significantly altered the decline in total functional capacity, but CoQ10 (with or without remacemide) resulted in a nonsignificant 13% decrease in the decline.

As noted by Young, although not significant, the 13% decline may be of interest for several reasons. The study was powered to detect only changes greater than 40%, and data from a Parkinson's disease trial indicate that 1200 mg/day, but not 600 mg/day, slows functional decline significantly. Chesselet added that her group just finished a trial in knock-in mice treated with CoQ10 from conception. The group observed a complete reversal of several behavioral

symptoms, using an open-field task, a very sensitive pole-descending task, and a rotarod task. Interestingly, only the lower of two doses tested was effective.

Plans to follow up on these results were discussed. Young noted that, based on the Parkinson's disease data, a new HD trial is now being set up in which doses of 1200 and 2400 mg/day will be tested. In addition, Chris Ross is working on setting up presymptomatic trials. He is examining safety and toxicity issues carefully because side-effects that might be tolerated by symptomatic patients, might not be acceptable to presymptomatic patients. Young added that CoQ10 has been tested, for other diseases, in doses of over 3 grams a day, and she was not aware of any associated toxicity even at these very high doses. Beal noted that CoQ10 analogs are also under investigation.

To extend her group's promising findings, Chesselet is now searching for a laboratory that can help with the pathological analysis of the mouse trial, including examination of the status of subcellular compartments. She also noted her interest in running additional mouse trials in which animals are treated post-symptomatically. Yang added that testing treatments administered at different ages in mice promises to be useful for guiding human trials.

Studies with creatine, an energy buffering molecule which is reduced in HD, are also promising. Young noted that creatine administration has dramatic effects on atrophy levels in mice and, anecdotally, patients are very positive about it. The compound is in phase II clinical trials, and a proposal is now being submitted for a phase III trial. (Doses of up to 30 g/day result in a linear increase in blood and brain levels. At approximately 40g/day, the levels fall off and bowel symptoms ensue).

Young noted that Steve Hersch and Diana Rosas have been evaluating symptomatic patients treated with creatine using brain imaging techniques. So far, they have observed beneficial effects as assessed by measurements of cortical thickness. Cortical thickness decreases with HD progression and correlates with functional capacity. In a couple of individuals treated with creatine, cortical thickness even increased. A larger trial is now being planned, followed by a trial with presymptomatic patients.

Beal noted that drugs that target the mitochondrial permeability transition (MPT)—a disruption in mitochondrial function associated with calcium dysregulation, abnormal caspase activity, and impaired energetics—are also under investigation for their therapeutic potential. Hazel Szeto at Cornell has designed small cell-permeable peptide drugs that are potent antioxidants and, some of them, protect against MPT, mitochondrial swelling, and apoptosis. The MPT protectors selectively accumulate in the inner mitochondrial membrane. Beal's group is planning to test these drugs in HD models. In addition, Bruce Kristal has identified several MPT inhibitors as part of a screening effort to find compounds for treating neurological disorders, in particular stroke. Promethazine emerged as a compound that has minimal effects on normal mitochondrial physiology, crosses the blood-brain barrier, has a long safety record, and mediates over 50% reduction in stroke volume in mice. However, Beal noted that, so far, promethazine has proven ineffective in HD mice. Chesselet suggested trying HD models that develop the disease slowly and early administration of the drug.

In addition, LaSpada described the glitazones as an extensive set of PPAR- γ agonists worth exploring. As previously mentioned, these compounds have been used as anti-diabetic agents, but they can also be neuroprotective. By boosting PPAR γ activity, glitazones enhance one branch of the PGC1 α signalling pathway, as well as downregulate inflammation. As noted by LaSpada, a recent study in a mouse model of amyotrophic lateral sclerosis indicates that oral treatment with pioglitazone protects from neurodegeneration, apparently by decreasing the

inflammatory response. In addition, as previously mentioned, pioglitazone stimulates Nrf2 activity. Although La Spada noted that Jenny Morton observed no effects after treating R6/2 mice with rosiglitazone, Beal said he observed a 14-15% increase in survival when adding pioglitazone to HD mice's food. This discrepancy might be explained, as noted by LaSpada, by rosiglitazone's inability to cross the blood-brain barrier. LaSpada added that testing PPAR α agonists might also be fruitful. However, Kuhn cautioned that these compounds cause liver toxicity.

Another compound, pointed out by Steve Finkbeiner, is AICAR, a potent activator of AMP-activated protein kinase. AICAR increases mitochondrial protein expression *in vivo* and has been described as neuroprotective because of its ability to block pro-oxidant and proinflammatory responses.

Action items

1. Assess PGC1- α activity in HD striatum and other tissues.
2. Examine Olsen and collaborators' microarray data to determine whether the expression of mitochondrial genes, particularly ones regulated by PGC1- α , is altered in HD.
3. Determine whether HD is associated with abnormalities in mitochondrial turnover.
4. Assess whether warming HD mice with thermoregulatory alterations can extend survival.
5. Determine whether body temperature alterations are associated with HD in humans.
6. Examine whether mitochondrial counts in peripheral blood cells may serve as a biomarker of disease.
7. Perform pathological examination of HD mice treated with effective doses of CoQ10.
8. Assess CoQ10's effects on HD mice treated at different stages of disease.
9. Test Szeto's MPT-protective peptides in HD models.
10. Assess effects of early administration of promethazine in HD mouse models that develop the disease slowly.
11. Extend glitazone studies to assess potential efficacy in HD.
12. Consider testing AICAR's effects on HD.

Additional therapeutic avenues

In addition to the various therapeutic targets discussed above, Young briefly summarized the status of other candidates in clinical trials. One of the most advanced compounds in the pipeline is tetrabenazine, a depletor of monoamines in nerve terminals which is prescribed for HD chorea in Europe, and in the process of being approved for HD in the United States. Tetrabenazine dramatically ameliorates HD chorea, and maintains its potency and efficacy even after years of treatment. The compound has a high selectivity for the vesicular monoamine transporter hVMAT2, which is predominantly expressed in striatal dopaminergic terminals. This is in contrast to other monoamine depletors, such as reserpine, which bind to both hVMAT1 and hVMAT2, and consequently also affect blood pressure and gut mobility. In addition, Young noted that tetrabenazine may have neuroprotective effects which Hersh is currently investigating in R6/2 mice.

Other candidates in clinical trials include the antibiotic minocycline—an anti-inflammatory agent that also inhibits caspase activity and the production of free radicals—which is now being evaluated in a futility study. Futility studies are designed to test treatments over a short period, in a small number of subjects, to determine if they should be abandoned or followed up by larger and longer studies. In addition, ethyl eicosapentanoic acid (EPA), an omega-3 fatty acid which has been tried, so far unsuccessfully, as Lax-101 in humans, is still being evaluated. Memantine, a non-competitive, low-to-moderate affinity N-methyl-D-aspartate (NMDA) receptor antagonist, is in safety and tolerability trials. The compound is approved for use in Alzheimer’s disease, but its benefits for HD remain unclear. Young noted that dosage studies in animal models of HD would be helpful.

Participants also discussed the potential of combination therapies. As noted by Young, HD alters many cellular and molecular pathways, such that combination therapies may ultimately prove to be the best way to treat HD. Indeed, as previously noted, combining cystamine and mithramycin appears to be a particularly promising approach.

Tools to accelerate flow through the HD pipeline

Cell and animal models of HD constitute the backbone of HD research. Participants discussed issues related to these models and ways to increase their usefulness. For example, Yang summarized his studies using the Cre/LoxP system to generate mice that express mutant huntingtin in either all neurons (pan-neuronal model), pyramidal cortical projection neurons (cortical model), or striatal neurons (striatal model). Phenotypic analyses of the pan neuronal model revealed hypoactivity similar to R6/2 mice, aggregates, gliosis, dysmorphic neurites, shrinkage and dark cell degeneration in the cortex and striatum. Both the cortical and striatal models, however, developed alterations much more slowly and never showed signs of gliosis. The cortical model resulted in some dark vesicles, but the striatal model showed no sign of dark cell degeneration. Mutant huntingtin localized to the nucleus in both models.

Based on these observations, Yang suggested a 2-hit model, in which both cell autonomous alterations and cell-cell interactions are critical. He noted that interneurons might be important players, either by failing to provide necessary support factors for other neurons, or by producing toxic stimuli. To continue examining the roles played by cell autonomous factors and cell-cell interactions, Yang is currently performing cell type-specific microarray analyses using fluorescence activated cell sorting (FACS) of tissues obtained from mice with specifically labelled cell populations.

Yang’s team has also developed a full-length human BAC model of HD in which mutant huntingtin can be switched off selectively in specific cell types. Participants were enthusiastic about the model’s potential for probing the roles of different cell types and their interactions in HD. In addition, the BAC model provides a new model of HD which Yang thinks mirrors human adult onset disease more closely than other models. When the mutant gene is expressed in all cells, subtle motor deficits (as assessed by rotarod performance) appear at 2 months of age, and become well-defined by 6 months. In addition, reduced brain volume can be measured by stereology at 12 months. Inclusions are barely detectable at 6 and 12 months, but become clearly visible at 18 months, with approximately 90% residing in the neuropil, and only 10% in nuclei. Neurite atrophy seems to occur in pyramidal neurons, followed eventually by cell loss. Behaviorally, the mice become hyperactive at approximately 12 months of age, coinciding with 30% striatal atrophy.

Whether these features reflect adult onset human HD better than other models of HD was subject to debate. Yang noted that the late appearance of inclusions, their predominance in the neuropil, and the late emergence of hyperactivity are fairly unique, and very similar to human HD. However, Chesselet considered that the BAC mice are not fundamentally different from other HD mice, and added that comparing mouse models is complicated. Variations in mouse strain make enormous differences in behavioral symptoms and disease progression is highly dependent on the number of CAG repeats. In addition, differences in mutant huntingtin expression levels and species (human or mouse) are also potentially important. To help resolve these questions, Young urged Yang and Chesselet to analyze the primary data jointly.

More generally, participants discussed problems associated with variations in mouse strain background. LaSpada suggested putting various huntingtin constructs into each of the commonly used mouse strains to enable researchers to confirm their results across various genetic backgrounds. Alternatively, several participants suggested performing back-crosses to avoid using inbred strains, while still having genetic purity, as noted by Bates. Yang stressed the importance of purity, noting the growing evidence for genetic modifier effects in humans. C. Johnson agreed, but worried about the complexity of obtaining these mice. Addressing this concern, Chesselet pointed out that Oswald Steward's group has performed a systematic comparison between FVB and Black 6 mice, and carried out back-crosses to generate F1 hybrids and several additional generations. The work, which promises to be a valuable resource for animal studies, will soon be submitted for publication.

A more general concern about inbred strains, as noted by Hughes, is that laboratory mice have been selected for rapid breeding, which results in the collection of genetic variations that probably affect the animals' overall health and lifespan, and their responses to experimental manipulations. Indeed, a recent study revealed that several so-called "longevity" mutations, which increase the lifespan of inbred *Drosophila* strains, fail to extend the lives of outbred flies. The "longevity" mutations appear to be correcting inbred defects of little physiological relevance to outbred populations.

Another issue of concern is the availability of various genetically engineered mice, as noted by Levine. For example, David Housman said there are approximately 300 lines of Gensat mice—transgenic BAC mice in which endogenous coding sequences have been replaced by the EGFP reporter gene—but Yang noted most are frozen and not readily accessible. Khachaturian suggested creating an NIH initiative, or collaborating with Jackson Labs. However, Levine noted that government efforts are usually burdened by bureaucracy, and commercial ventures are too expensive. Instead, Levine proposed that the HDF and/or other HD foundations help distribute these resources. Foundations, he noted, are efficient and have a better understanding of researchers' needs. Wexler proposed setting up a committee to discuss this possibility.

Participants also briefly discussed the availability of HD ES cells. J. Johnson asked whether human HD ES cells could be used to develop new cell models of HD. Summarizing the conclusions of a recent workshop on this topic, C. Johnson noted that ES cells isolated from HD embryos are available, but they have limitations. Alternatives include knocking in CAG repeats of different lengths into huntingtin genes of normal human ES cells, or transplanting nuclei from HD patients into normal human ES cells. An advantage of the latter strategy is the available information on the mutation's clinical phenotype.

Action items

1. Continue Yang's studies dissecting the roles of cell autonomous processes versus cell-cell interactions in HD pathology:
 - a. Use FACS and microarray analyses to track cell-specific responses
 - b. Use new BAC mouse model to delete mutant huntingtin from specific cell populations
2. Analyze differences between Yang's BAC model and other HD models (Chesselet and Yang)
3. Use Steward's recent study of FVB and Black 6 mice as a resource to address genetic background issues.
4. Discuss possibility of establishing a mouse repository managed by the HDF and/or other HD foundations.

Some final thoughts

The need for improving communications between basic scientists and clinicians emerged as a top priority. As noted by Young, there is a paucity of basic experiments to directly help guide and design clinical trials. For example, systematic studies of dosages and blood-brain barrier permeabilities are needed for many candidate compounds. Part of the problem, noted Young, is that basic scientists are often not motivated to perform these characterization studies. An alternative is to hire companies, but finding reliable ones can be challenging. Davidson pointed out that much might be accomplished if the leaders of clinical studies inform the scientists of the data they need, as well as of the data they have already gathered (e.g., imaging data, neuropsychological data, biomarker assays, pathology data). Young noted that an initiative that could facilitate this process is the Systematic Evaluation of Treatments for HD, SET-HD. The project's goal is to help prioritize interventions for testing in pre-clinical and clinical trials by systematically compiling clinically relevant HD data (www.huntingtonproject.org).

Another major issue is the establishment of validation criteria for testing candidate compounds in clinical trials. Lemke considered that HD research has moved past the exploratory phase in which it is useful to investigate many approaches with few constraints, and is now at a point that would benefit from reaching a consensus on specific assays to guide therapeutic development. Several participants agreed, but noted some associated difficulties. One problem, said Young, is that there are many conflicting opinions in the community. For example, Lindquist and Kuhn stressed the importance of obtaining solid animal data to design effective clinical trials, and Kuhn added that a mechanistic understanding of candidate therapies should be required. However, Young noted that many clinicians are anxious to provide treatments quickly and are thus reluctant to wait for these data, particularly when the compounds to be tested are known to be relatively safe.

Paganetti considered that the major question to resolve is deciding which screening assays to use. Kuhn noted that it will also be important to establish criteria to decide when to stop pursuing less promising candidates. Unfortunately, as pointed out by Thompson, it is difficult to resolve these questions because it is still unclear which assays best reflect the human disease.

The need for better biomarkers of disease was also noted. Kuhn underscored the importance of obtaining more robust markers, in both humans and animals, that can be assessed relatively quickly. Young pointed out that several promising imaging studies are in progress.

Indeed, as previously noted, Rosas and Hersch are using cortical thickness as a marker to track the effects of creatine. In addition, several searches for biochemical markers of HD are underway.

Another issue discussed was the importance of considering disease progression when designing experiments and clinical trials. As noted by Levine, HD changes dramatically as a function of time. For example, NMDA currents exhibit very different behaviors at various stages of disease. In several cases, such as when targeting glutamate receptors which disrupt entire neuronal networks and are altered perhaps even before birth, early intervention will probably be critical. As noted by Young, full-length mouse models which develop the disease more slowly might help dissect such timing questions and help guide future clinical trials. It will also be important to consider compounds' side-effects when determining the time to begin treatments. As noted by Wexler, some compounds may present toxicity problems when administered chronically for long periods. Also, side-effects tolerated by symptomatic patients might not be acceptable to presymptomatic patients.

Khachaturian noted that another point to bear in mind is patient response heterogeneity. When evaluating the effects of candidate compounds it will be important to analyze the data so that subgroups of responders can be identified. Compounds that benefit even only 10% of a patient population can be very valuable.