

Hereditary Disease Foundation

Fast Tracks to Lead Compounds

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The search for compounds to treat Huntington's disease (HD) can not proceed fast enough. Such was the sentiment inspired by those coping with HD, who generously shared their experiences at the January 2001 Hereditary Disease Foundation workshop. A 27-year-old woman, for example, described how her careers in architecture and modeling were truncated by the progressive symptoms of HD - uncontrollable movements and declining cognitive faculties. And a father bravely recounted how his son committed suicide only a few weeks earlier, to avoid the fate of his sister who is now living on a feeding tube as a result of HD.

The time seems ripe to begin developing treatments to lessen, or even eliminate, such suffering, but much work lies ahead. Much of the basic science of HD remains obscure. Little is understood, for example, of how expanded CAG repeats in the huntingtin gene lead to a vast array of cellular dysfunctions, and ultimately death, in selective regions of the brain. It is still unclear if a single molecular event triggers a cascade of downstream effects or if, alternatively, an initial event affects many cellular processes simultaneously. It is also uncertain whether mutated huntingtin mediates its primary effects by directly damaging the medium spiny cells in the striatum or by disrupting the function of the cortical neurons that project to the affected striatal cells. And although it is clear that a central aspect of HD pathology involves the formation of protein aggregates, the causal links between the size, composition, and density of these aggregates and the progression of disease are just beginning to surface.

Participants at the workshop recognized and discussed approaches to resolving these uncertainties. But they also noted that focusing efforts on a few of the molecular clues now available might yield therapeutic candidates, even at this early stage of research. Several strategies for screening compounds that specifically target long stretches of poly-glutamine repeats were presented. Assays to monitor the molecular structure, aggregation properties, expression, toxicity, proteolytic cleavage, and nuclear transport of polypeptides containing large poly-glutamine repeats emerged as promising options for finding and characterizing potentially therapeutic drugs.

From these screens, participants predicted that hundreds of candidate compounds are likely to emerge. The next challenge will be to whittle down the list for testing in animal models and, ultimately in clinical trials. By identifying rate-limiting steps, such as the availability of mice and the recruitment of neurologists, participants suggested ways to optimize the funneling process. They suggested outsourcing toxicological and pharmacokinetic studies, for example, to making testing in mice more manageable. And to increase the power of clinical trials, they proposed identifying improved genetic, pathological, and behavioral biomarkers of disease.

Basic research: some unresolved questions

The chicken-and-egg problem: What is the temporal sequence of events in HD?

A fundamental problem in HD research is understanding the sequence of events that links expanded CAG repeats to behavioral

abnormalities and, ultimately, death. As pointed out by Michael Levine, a growing list of HD-associated defects is being compiled, yet very little is known about how these abnormalities are linked to each other through cause and effect. It is still unclear, for example, how the alterations in the electrophysiological properties, the sensitivity of NMDA receptors, and the calcium responses of striatal cells in HD mouse models causally relate to each other. It is possible that mutated huntingtin sets off a cascade of events that sequentially lead to these multiple alterations or, alternatively, that the mutated protein directly affects various processes simultaneously.

Clues from gene expression studies

Searching for early alterations that could shed light on the progression of HD, James Olson described his team's search for changes in gene expression. Monitoring approximately 6000 genes in the striata of 12-week-old R6/2 mice, Olson found that the expression of a small subset of genes, less than two percent of the total, were affected by HD. Most strikingly, the activities of genes involved in signal transduction pathways seemed to be blunted. Trying to elucidate the progression of these transcriptional changes and distinguish primary changes from secondary and compensatory changes, Olson has now examined the profiles of younger animals. Surprisingly, even in four-week-old animals, he found alterations in the expression of each of the categories of genes that were altered in the 12-week-old animals. The magnitude of the changes increased with age but, qualitatively, the changes were very similar. At first blush, these results seem to support the possibility that mutated huntingtin has simultaneous effects on multiple cellular processes. However, as pointed out by Ethan Signer, huntingtin is expressed approximately two weeks *before* birth, such that numerous changes downstream of the mutation are likely to have occurred by the time the mice are four weeks old. Acknowledging this limitation,

Olson added that in yeast, alterations in gene expression can be observed as early as 24 hours after the induction of the expression of mutated huntingtin.

Various options to resolve this issue were proposed. Mary Kennedy, for example, suggested monitoring gene expression very early in development, perhaps even in the stem cells that ultimately give rise to striatal neurons. Olson described an alternative strategy, which he is now beginning to use: monitoring gene expression in mouse models that express huntingtin in an inducible fashion. So far, he has detected *more* changes at earlier time points - such as one week after induction - than at later stages. The results suggest the brain enlists compensatory mechanisms to minimize the disruption induced by the mutated protein. Carl Leventhal raised the question of whether the onset of disease symptoms could be due to the wearing off of these compensatory mechanisms, or alternatively, to reaching a disruption threshold above which the brain is no longer able to compensate effectively. Olson added that the compensatory changes probably contribute to the disease process itself. Pointing out a potential limitation of the inducible mouse models, however, Stephen Dunnett noted that turning on a gene in an adult animal may not effectively reproduce what occurs when the gene is naturally activated during development, since the adult and embryonic brains are drastically different environments.

In order to refine the search for the sequence of events that underlie the disease, and ultimately pinpoint the pathology's primary cause, participants suggested examining different groups of cells separately. Kenneth Fischbeck, for example, proposed that the disease could progress in a mosaic fashion. Olson agreed, noting that examining the dorsal versus the ventral regions of the striatum may yield valuable clues regarding progression. He also mentioned he was beginning to test the

feasibility of performing fluorescence-activated cell sorting (FACS) to measure gene expression profiles in distinct cell populations.

The microarray experiments have helped establish that changes in gene expression are an early event in the progression of HD, but how these alterations arise from an expanded stretch of glutamines within huntingtin remains uncertain. David Housman noted that the presence of a poly-glutamine stretch in a protein seems to predispose it to becoming incorporated into huntingtin aggregates. Since many transcription factors sport poly-glutamine repeats, they may become sequestered in these aggregates, or their functions may be disrupted through interactions with mutated huntingtin. Leslie Thompson has found, for example, that huntingtin exon 1 can interact with p53 and the transcriptional co-activator CREB binding protein (CBP). The nature of these interactions seems to be affected by the length of the huntingtin poly-glutamine repeat. Robert Hughes, on the other hand, described studies suggesting mutant huntingtin may affect the histone acetylation. When he expressed mutated exon 1 in yeast, he observed a repression in the transcription of several genes. He was then able to de-repress at least two of the most severely affected genes with trichostatin A, an inhibitor of histone deacetylation. Olson cautioned, however, that trichostatin A, itself causes massive changes in gene expression.

Clues from genetics

The search for modifier genes may be another source of insights into how mutated huntingtin causes disease. Nancy Wexler pointed out that the transmission of CAG repeats from generation to generation in humans is very variable, but often consistent within single genealogical lines, suggesting the existence of modifier genes that affect the process. In addition, the age of onset of HD correlates not only with repeat length, but with the age of onset of the disease in a patient's

siblings. Working in Housman's lab, Michael Andresen will be conducting a genome-wide search for such modifiers. Their identification promises not only to improve the power of clinical trials by enhancing the predictability of disease onset (see *Biomarkers and surrogate markers of disease*), but may also reveal key cellular components that interact directly or indirectly with mutated huntingtin, thus shedding light on the molecular mechanisms underlying the disease and providing new targets for drug development.

Human genetics studies discussed by Blair Leavitt promise to provide similar mechanistic clues. Michael Hayden's group, for example, are analyzing the genomes of patients suffering from HD-like symptoms who lack expanded CAG repeats in their huntingtin genes. Having identified a mutation that is associated with such a condition, Leavitt predicts that the newly found locus may be involved in a pathway that is normally disrupted by mutated huntingtin. Greg Lemke noted, however, that an alternative possibility is that the mutated gene damages a similar set of neurons as those affected by mutated huntingtin, but does so through an unrelated mechanism.

Medium spiny cell death: Murder or suicide?

In addition to dissecting the molecular links between mutated huntingtin and other cellular components, HD researchers must dissect the inter-cellular links between brain regions that underlie disease progression. It remains uncertain whether the main targets of HD, the medium spiny cells of the striatum, are damaged directly by expressing mutated huntingtin, or indirectly, through changes in their cortical inputs caused by the mutated protein. As expressed by Signer, is the cause of death of the medium spiny neurons suicide or murder?

Several experiments in progress are addressing this question. As described by

Signer, at last summer's HD symposium Dan Goldowitz and Anton Reiner reported pathological analyses of mosaic animals sporting cortices expressing mutant huntingtin and striata expressing wildtype huntingtin, as well as animals with the opposite combination, suggesting that the cortex might play a key role in striatal pathology. However, Dunnett has observed that when wildtype striatal cells are transplanted into R6/2 mice, the wildtype cells remain healthy and inclusion-free up to eight weeks after transplantation. One possibility, suggested by Lemke, is that both murder and suicide might contribute to striatal cell death. Dunnett is now planning to extend his studies to other mouse models, including Michael Hayden's YAC model. To complement these studies, Kelsey Martin suggested using organotypic slice cultures, which can be kept alive for weeks and are much more amenable to manipulations.

Why are striatal medium spiny cells so vulnerable?

Regardless of whether striatal spiny cell death is the result of murder, suicide, or both, the reason for their vulnerability remains elusive. Some researchers have proposed that their susceptibility stems from their high energy expenditure and consequent sensitivity to mitochondrial dysfunction. As explained by Leavitt, for example, accidental ingestion of mildewed sugar cane by humans resulted in the acute onset of motor dysfunction and striatal degeneration similar to HD. The mold toxin 3-nitropropionic acid was identified as the cause of the syndrome and was found to act as an inhibitor of succinate dehydrogenase. Subsequently, the toxin has been used to cause striatal neurodegeneration in mice and rats as an animal model of HD. In addition, the sensitivity to anoxia appears to be greater in the medium spiny neurons of the striatum than in cells from many other brain areas. However, Charles Wilson noted that the evidence for this greater

consumption is not well established and that spiny cells do not fire many action potentials, as expected of neurons with high energy requirements.

There are several other ways to explain spiny cell vulnerability. Dunnett, for example, noted that spiny cells are rare in that they receive two excitatory inputs, glutamatergic and dopaminergic, and there is evidence that the dopaminergic inputs contribute to the toxicity of the glutamatergic inputs. But Wilson noted that dissecting the toxic contributions of each was difficult because when dopaminergic inputs are lesioned, the glutamatergic inputs pull away.

Another possibility pointed out by Kennedy is that striatal cells may harbor a unique complement of proteins involved in regulating intracellular calcium. As noted by Levine and Leavitt, the calcium responses of striatal cells in mouse models of HD are altered as compared to controls. For example, Levine has observed in pre-symptomatic R6/2 mice an increased calcium influx in response to depolarizing voltages. Yet once the animals begin to express behavioral abnormalities, he detects a *decreased* calcium influx in response to the same type of stimulus. Either a subpopulation of high-responding cells are dying during disease progression or a single population of cells is changing its response characteristics. Kennedy suggested using calcium-sensitive dyes to examine these behaviors in greater depth. To obtain temporal information, she proposed using statistics to analyze the responses of large groups of cells at distinct time points. Tobin added that the use of two-photon microscopy could provide a complement to this approach by providing a gentle way of monitoring single cells. Some researchers have already begun to use calcium imaging in cell culture systems. Thompson, for example, is setting up a system to image PC12 cells harboring an inducible copy of mutated huntingtin. Kennedy recommended, however, that she switch to primary mouse

neurons, since PC12 cells lack several neuronal hallmarks such as the ability to make synapses.

Participants also discussed the possible role of somatic repeat variability in determining the vulnerability of different brain regions to HD. Gillian Bates, for example, pointed to the work of Peggy Shelbourne showing that the CAG repeats in the striatal cells of a knock-in mouse model of HD seem to become dramatically unstable as the mice age. And as described by Wexler, Norman Arnheim recently detected repeat variability in human cortical tissue. Leavitt stated that somatic instability has been shown to be highest in those regions of HD brain corresponding to the regions of earliest pathology and that this early study suggested that mitotic instability might contribute to the selective neuronal death in HD. He also suggested that use of single-cell PCR might help resolve the debate over whether the instability occurs in mitotic (glia) or post-mitotic (neurons) cells. Repeat instability is thought to be replication-dependent, such that finding instability in neurons would be particularly intriguing. But Bates noted that even if the instability maps to glial cells, the observation would be striking since, on average, glial cells turn over only rarely. Participants agreed that Arnheim should try to compare repeat variabilities across as many tissues as possible in order to discern whether particular subsets of cells are preferentially affected. Ann Graybiel stressed the importance of using clearly identifiable landmarks when extracting pieces of human brain tissue in order to ensure reproducible results.

Huntingtin aggregates: Size matters

The role of huntingtin aggregates in the disease process has long been a subject of intense debate. But as noted by several participants, the emerging concept that not all aggregates are the same, and that they can differ radically in their degree of toxicity, may help reconcile apparently contradictory results.

Susan Lindquist, for example, noted that her studies in yeast suggest that large aggregates are considerably less toxic than smaller ones. She speculated that large aggregates can become toxic when they are broken down into smaller pieces, perhaps due to the increase in surface area. Fischbeck noted that a similar relationship between size and toxicity has been observed in mammalian cells, and that experimentally altering the ratio of larger to smaller compounds can correspondingly affect toxicity.

In addition to size, other aggregate properties may contribute to their effects. Lindquist, for example, described observing similar-sized aggregates that appear to be packaged differently as assessed by their solubilities in detergents such as SDS. In addition, the composition of aggregates may vary depending on the surrounding cellular components. As mentioned earlier, proteins containing poly-glutamine stretches seem to readily incorporate into growing huntingtin aggregates.

A striking example of the likely importance of these distinctions was provided by Housman's and Thompson's experiments using peptides to suppress aggregation. The researchers designed a peptide containing an alpha-helical loop of the TATA-binding protein, flanked by stretches of 25 glutamines on either side. Previous experiments, using GFP to track poly-glutamine constructs, had established that peptides consisting of 25 glutamines readily incorporate into aggregates. So Housman and Thompson expected that the glutamine repeats in their designed suppressor would target the alpha-helical loop to aggregates, coating the aggregates' surfaces with a protein structure not conducive to further aggregation.

When Housman and Thompson monitored the effects of the suppressor on the gross appearance of aggregates in cells, they did not detect major changes: the cells harbored aggregates

throughout, similarly to control cells not expressing the suppressor. But the effects of the suppressor on toxicity were dramatic. The researchers used the UAS/GAL4 system to control the expression of the peptide suppressor and a peptide containing a stretch of 108 glutamines (Q108) in *Drosophila*. Without the suppressor, all flies expressing the Q108 peptide died during larval development. Yet when expressing the suppressor, 60-70% reached adulthood. An analysis of photoreceptor degeneration revealed that the suppressor was able to specifically rescue neurons from poly-glutamine toxicity.

Housman and Thompson are now using differential tags to analyze more carefully the cellular distribution of the suppressor and its relationship to the poly-glutamine aggregates. As expected, the suppressor co-localizes with aggregates, but the scenario is complex: aggregate size and composition ratio, (of suppressor to aggregate-forming peptides) vary depending on several factors, such as the expression levels of each peptide. Thompson noted that the inhibitors might be acting not only to inhibit aggregate growth, but to delay aggregate formation.

Key to understanding aggregate formation and its consequences is understanding the role played by poly-glutamine length. Using *in vitro* experiments to monitor the aggregation of poly-glutamines, Ronald Wetzel identified kinetic and thermodynamic parameters of aggregation that correlated with polypeptide length. He observed, for example, that solutions containing long polypeptides begin to form aggregates more quickly than solutions of short polypeptides. Also, after reaching equilibrium, aggregates composed of longer polypeptides have incorporated more monomers than those of shorter polypeptides. Lindquist noted that her

experiments in yeast also suggest that the length of poly-glutamine repeats affects the maximum level of aggregation reached at a given protein concentration. An open question, however, is whether polyglutamine length also affects the types of proteins included in *in vivo* aggregates. Lindquist suggested using protein chips to screen massively for proteins that preferentially interact with long poly-glutamine stretches.

Several researchers are also examining the role of context on aggregate toxicity. To study aggregate distribution within cells and how it affects toxicity, for example, Paul Ko Ferrigno is expressing GFP-tagged huntingtin in various yeast mutants deficient in nuclear transport. Leavitt, on the other hand, is exploring the role of wild-type huntingtin and how the loss of wild-type huntingtin function may play a role in HD. Although the mechanism remains uncertain, wild-type huntingtin appears to decrease the toxicity of mutant huntingtin, and preliminary studies suggest that wild-type huntingtin also protects neurons from excitotoxic cell death *in vivo*. He noted that a recent study by Scott Zeitlin and colleagues showing that inactivation of wildtype huntingtin in the brains of healthy mice results in progressive neurodegeneration is consistent with these findings. Brent Stockwell asked whether overexpression of wildtype huntingtin might thus have therapeutic potential. But many uncertainties remain. Wexler noted, for example, that neither the age of onset nor the severity of symptoms of the few homozygote patients that have been studied to date seems to be strikingly different from those of heterozygotes.

Screening for lead compounds

Given the uncertainties surrounding the nature and causal relationships of the various pathological changes associated with HD, Signer and Lemke stressed the need to develop screens for therapeutic candidates that target the disease's one clear, primary source of trouble:

expanded poly-glutamine repeats. Participants discussed strategies to screen for compounds that selectively bind, disrupt the structure, or interfere with the aggregation of long poly-glutamine repeats. Keeping close to the poly-glutamine target, but aiming their screening strategies one step upstream, some participants presented assays for identifying compounds that inhibit the production of toxic huntingtin polypeptides. Conversely, aiming their searches one step downstream, others proposed searching for compounds that inhibit the early effects of the expression of expanded poly-glutamine repeats.

Aiming directly at the poly-glutamine repeats

Screening for compounds that preferentially bind expanded repeats

In search of structures that preferentially bind mutated huntingtin, Ko Ferrigno described screening a combinatorial library of 3×10^9 aptamers. Using thioredoxin as a scaffold to constrain the shapes adopted by the aptamers, he scanned the library for molecules that interact with a fragment of huntingtin including exon 1 and a stretch of either 17 or 68 poly-glutamine repeats. Three types of aptamers emerged from the screen: a set that bound equally well to the huntingtin bait whether it contained 68 or 17 poly-glutamine repeats, a set that bound preferentially to the bait with 68 repeats, and a set that bound preferentially to the bait with 17 repeats. Sequencing eight of the candidates that bound preferentially to the 68-repeat bait, Ko Ferrigno has identified a conserved sequence shared by six of these aptamers: a tryptophan followed by a leucine. Based on mutation studies, the tryptophan appears to be necessary, although not sufficient for binding.

To hone the abilities of his promising candidates, Ko Ferrigno described several approaches he is now setting up. For example, to improve the affinity of his aptamers, and achieve binding in the nanomolar range, he

plans to use random PCR mutagenesis. He also wants to pinpoint the aptamers' binding sites by using smaller huntingtin baits, as well as trim the aptamers, which are currently 20 residues long. Before undertaking this pruning effort, however, he plans to test the 20-mers' ability to reverse toxicity. Furthermore, to examine the effects of specifically targeting huntingtin residing in the nucleus, he plans to add a nuclear localization signal to his candidates. He is also interested in fusing ubiquitin to his aptamers, hoping it might help direct aptamer-huntingtin complexes to proteasomes for degradation. However, as noted by Olson, huntingtin is often ubiquitinated **in vivo**, and yet it is not degraded.

Similarly searching for compounds that recognize long stretches of poly-glutamine repeats, Shiva Malek and Brian Pollak presented a screening strategy that monitors the ability of an antibody to bind to its target: long poly-glutamine repeats. The strategy relies on labeling an IgM antibody, developed by Paul Patterson's group, which preferentially binds to huntingtin harboring long stretches of glutamines, with a fluorescent tag to monitor its interactions with GFP-tagged huntingtin exon 1. The idea is that when the antibody binds to the exon 1 construct, the fluorescent tags are brought into close proximity allowing fluorescence resonance energy transfer (FRET) to occur. In theory, compounds that bind to the poly-glutamine stretch of exon 1 will block the binding of the antibody, and thus prevent FRET. The researchers have successfully purified exon 1 constructs containing 25 and 47 glutamine repeats, but they have yet to test energy transfer interactions with the antibody.

A similar concept is being developed by Hughes who is also using the Patterson antibody to screen for compounds that interact with long poly-glutamine stretches. However, Hughes is relying on a yeast-two hybrid system, instead of FRET, to monitor the antibody's interactions. In order to avoid making multiple constructs to

express each of the antibody chains, and to ensure the antibody's integrity within the reducing environment of the yeast cell, he is using a single-chain form of the antibody in which the chains are held together by covalent bonds.

Screening for compounds that interfere with aggregation

Yet another set of screening strategies seeks to monitor aggregation and the conformational changes that underlie its initiation. Malek and Pollak described the design of a system that relies on FRET to track conformational changes. The researchers designed a construct that contains a stretch of either 25 or 45 poly-glutamine repeats, flanked by a donor and an acceptor fluorescent protein on either side. The prediction was that when the poly-glutamine stretch adopted a folded structure, the donor and acceptor molecules would be brought together and FRET would occur. A lack of concentration-dependence in the reaction would serve as a control, indicating FRET was occurring as a result of intra-molecular, rather than inter-molecular, interactions. Alternatively, as suggested by Ko Ferrigno, a mixture of constructs with donors at both ends and acceptors at both ends could be used as a negative control.

But preliminary results suggest the assay will need revisions. Both the 25- and 45-repeat polypeptides appear to exist as random coils, as assessed both by the absence of FRET and by circular dichroism. One possibility is that, within these artificial constructs, the repeats are not constrained as they usually are by the full-length huntingtin protein. Ko Ferrigno suggested using a scaffold protein, such as thioredoxin, to circumvent this problem. Alternatively, several participants proposed creating more physiologically relevant constructs -- including exon 1, for example. Malek said they were working on it, but that such constructs were often hard to express, not

very soluble, and overly susceptible to degradation.

Participants also noted the potential importance of environmental conditions. Graybiel asked if the assay could be adapted to cells, and Martin suggested adding neuronal lysates to the reaction. But Jeffrey Kelly noted that to avoid false positives and streamline the screen, it was probably best to start with a clean, highly defined system. Lindquist suggested attaching the polypeptides to a chip to enhance throughput. Malek feared that immobilization might block conformational changes from occurring, however, even if the molecules were tethered at only one end.

Furthermore, the inability to observe FRET in preliminary experiments may be unrelated to the technical details of the assay's set-up. Conformational changes that trigger aggregation may, in fact, occur only very rarely. Alternatively, aggregation might *depend* on a random coil structure. If such is the case, then the screen could be used to search for compounds that induce FRET, rather than abolish it.

An alternative strategy, discussed by Housman and Stockwell, relies on monitoring the process of aggregation itself by measuring the fluorescence of a GFP-tagged construct of huntingtin developed by Michael Sherman. The construct fluoresces in an aggregation-dependent manner and can be expressed in yeast, such that toxicity and aggregation can be measured simultaneously. Monitoring toxicity is particularly important since, as pointed out by Wetzel, a compound that inhibits the growth of large aggregates by fostering the buildup of small ones, for example, could enhance the disease process rather than inhibit it. So far, this assay has revealed 13 compounds that appear to suppress aggregation by a factor of four or five.

Raising the issue of drug permeability, Stockwell noted he has had limited success with

this assay and suspects that many compounds may be unable to reach their targets within yeast cells. This problem has been circumvented in other systems by using yeast mutants deficient in their ability to pump out exogenous substances. Stockwell's problem, however, has persisted even when using these mutants.

A clever alternative to monitoring aggregation directly was presented by Hughes. He searched for a protein whose function would be disrupted when fused to a fragment of huntingtin containing expanded repeats, but not when fused to a wildtype fragment. After testing several proteins, he discovered that yeast cells expressing alpha-tubulin fused to the mutated huntingtin fragment are severely damaged and eventually die, while those expressing the same protein fused to a wildtype fragment appear healthy. Using this system to screen for compounds that rescue cells expressing the expanded construct, Hughes has obtained promising preliminary results. For example, he recently obtained eight hits from a screen of GNC plant extracts. Once he confirms these hits, he plans to begin separating the extracts into individual components.

Upstream targets

An alternative to targeting the poly-glutamine repeats directly is to target their production. Malek and Pollak described an assay, which promises to identify compounds that preferentially inhibit the generation of mutated huntingtin. To monitor the synthesis of control and mutated huntingtin proteins simultaneously, the researchers plan to use ecdysone inducible promoters to express two constructs in PC12 cells: huntingtin exon 1 with 25 glutamines fused to GFP, and huntingtin exon 1 with 103 glutamines fused to a red fluorescent protein. To avoid integration site variability, Lindquist suggested placing both constructs on a single plasmid. The researchers hope that by tracking decreases in the ratio of red to green fluorescence, they will identify

inhibitors of the processes that lead from the transcription to the generation of toxic protein, including mRNA processing and translation. They hope to avoid general inhibitors of protein production, by restricting their search to those compounds that do not appreciably affect production of the control protein –exon 1 with 25 glutamines.

Housman noted that targeting CAG repeats within mRNA, rather than poly-glutamine repeats within proteins, could be a potentially powerful strategy. RNAs often adopt sequence-specific structures which can be recognized by other molecules, and which could thus potentially serve as specific binding targets for therapeutic compounds. Dealing directly with mRNA would avoid the complications of aggregates - one could even develop constructs bearing CAG repeats within untranslated regions of mRNA to bypass working with the huntingtin protein altogether. Dennis Choi added that targeting mRNA might yield compounds with therapeutic properties beyond HD. But in order to examine these possibilities, Housman pointed out that Malek and Pollak needed new constructs - the ones they are currently using contain CAG repeats mixed with CAA repeats. Although these constructs are useful for stabilizing the expression of mutated huntingtin in bacteria, their RNA structures are probably very different from those containing pure CAG repeats.

Yet another possibility to search for compounds that block the production of toxic huntingtin products is to monitor the cleavage of huntingtin and its ability to get into the nucleus. Malek described the design of an exon 1 construct fused to a nuclear export signal on one side, and to a transcription factor that activates the expression of a beta-lactamase gene on the other. Only when exon 1 is cleaved, and thus separated from the export signal, can the transcription factor move into the nucleus and activate production of the enzyme, whose

activity can then be tracked by the generation of a fluorescent product. Searching for compounds that block this activation could yield new protease inhibitors, as well as inhibitors of nuclear transport.

A lack of basic knowledge regarding huntingtin proteolysis, however, could present some difficulties. As Signer pointed out, it remains unclear which cleavage sites play important roles *in vivo*. Thompson encouraged the researchers to use larger pieces of huntingtin, but Wetzel cautioned that expressing a large piece could expose a wealth of protease sites that may not be relevant to the disease process. Lemke added that protease inhibitors can disrupt many cellular functions and, thus, only very specific inhibitors are likely to have therapeutic potential. As pointed out by Leavitt, Cheryl Wellington has presented proof that huntingtin is definitively cleaved by caspases *in vivo*, but he admitted that other huntingtin cleavage events may occur or be more relevant for HD pathogenesis. He also stated that both mutant and wild-type huntingtin are cleaved, but it is as yet unknown whether cleavage of wild-type huntingtin differs from cleavage of mutated huntingtin. The consequences of cleaving the two proteins are clearly very different, but the cleavage patterns might be similar or identical. Finally, participants stressed the importance, and potential difficulty, of identifying an appropriate cell line to conduct the assay. Pollak said they were planning on using a human neuronal line, but Olson suggested screening several cell systems, including rat striatal cells, for example, to establish which ones generate peptide patterns that are more likely to be relevant to the disease process.

Downstream targets

Screening for targets downstream of huntingtin can be risky because of the gaps in knowledge surrounding the chain of events triggered by the primary mutation. Nevertheless, a few such approaches are

yielding promising leads. Olson, for example, has begun analyzing the changes in gene expression that mark the early stages of the disease in search of potential therapeutic targets. Although scores of cell culture-based assays will probably be required to whittle down the hundreds of potential intervention sites revealed by his microarray experiments, Olson has identified one particularly intriguing target. He has found that the retinoic acid receptor, RXR gamma, and a retinol binding protein are expressed at decreased levels in the striata of R6/2 mice. In addition, over 20% of the genes that are downregulated in the striata of these mice contain retinoic acid response elements. Interestingly, the striatal neurons that are exposed to the lowest concentrations of retinoids are those that degenerate first in HD. In addition, retinoid levels steadily decrease during aging. Taken together, these results suggest that retinoids may have therapeutic potential. To test this proposal, Olson is monitoring the effects of retinoic acid in mouse models of HD.

Another intriguing clue revealed by Olson's experiments is the increased expression of inflammatory response genes in 12-week-old R6/2 mice. Speculating that inflammation may contribute to the pathology of HD, Olson suggested conducting a more thorough examination of the effects of immunosuppressants in clinical trials, where they have been used to minimize transplant rejection. Dunnett pointed out, however, that trials in which Parkinson's disease patients received transplants of fetal primary tissue had revealed that patient outcome was correlated with transplant efficacy, but not with immunosuppressive treatment. Levanthal added that recent trials testing the effects of anti-inflammatory drugs on the progression of Alzheimer's disease have yielded discouraging results. But what applies to other neurodegenerative diseases may not apply to HD. Leavitt noted that human

neuropathological data suggest inflammation, including microglial activation and upregulation of cytokines, is an important feature of HD. To examine Olson's proposal further, Signer proposed using an immuno-compromised mouse, such as a SCID mouse, to create a transgenic model of HD.

Moving candidates through the pipeline: from hits to clinical trials

The emerging wealth of screening approaches bodes well for the development of treatments for HD. But optimizing the strategy for funneling screening hits into animal model testing, and ultimately into clinical trials, promises to be challenging. Pollak estimated a 0.1% hit rate for any given screening assay, thus generating approximately 500 candidates when screening 500,000 compounds. As pointed out by Tobin, this output must be massively pared down before selecting candidates for clinical trials. Analysis of the chemical structures of candidates will probably provide a first reduction in the pipeline. As noted by Carl Johnson, candidates will probably fall into only a few categories, such that the top representatives of each group can then be selected for further testing. Pollak added that a key step following the initial screening assays would involve recruiting chemists to improve the affinities of lead compounds. Although this step is time consuming and expensive, it often enhances efficacy by a factor of one hundred.

Animal testing

The testing of candidates in animal models emerged as a probable rate-limiting step. In particular, performing the necessary pharmacokinetic and toxicity studies promises to be a difficult and time-consuming process. Johnson noted that some relevant information may be gleaned from the compounds' chemical structures. But the bulk of the work will necessarily be experimental. Several

participants suggested delegating these studies to industrial contractors with FDA experience.

Although toxicity studies might be more efficiently handled by industry, Olson stressed that efficacy studies could benefit from continuing pre-clinical work in academic labs. He noted that the tenacity of academic investigators has already been critical for the advancement of some compounds that are now entering clinical trials, such as creatine. Furthermore, spending more time working with the mice should yield basic information about the models and lead to the development of more informative measures of improvement. Finally, he noted, conducting pre-clinical studies often provides a healthy source of motivation for members of academic labs.

In order for academic labs to conduct murine drug studies, Olson enumerated the following requirements: 1) gaining advice from industry-based experts, 2) setting up an accessible, reliable mechanism for testing cerebrospinal fluid and serum drug levels, and 3) establishing uniform testing procedures and quality control standards. Olson predicted that even simple standardization measures, such as recording data into the same colony management software and measuring task performance using standard protocols at similar intervals, would greatly enhance investigators' ability to compare data generated by multiple labs. Finally, he proposed that a hands-on workshop, at a site with extensive experience analyzing HD mice, could serve as a first step towards this goal.

Several participants suggested additional ways in which the HDF could facilitate animal drug testing. Ko Ferrigno, for example, suggested the HDF establish a battery of tests for the validation of candidates, and Pollak proposed the foundation set up guidelines for rating compounds. But Bates and Kelley cautioned that drawing up too many

specifications at this early stage could overly constrain future research.

To help alleviate the animal testing bottleneck, Stockwell proposed developing systems for conducting high throughput screening in mice. He suggested clustering candidate compounds to run multiplex assays using activity read-outs orthogonal to toxicity. Several participants noted, however, that currently available read-outs were far from optimal. And Wetzel cautioned that employing such, screens, in the absence of pharmacokinetic data, might prematurely eliminate promising candidates, such as those unable to cross the blood-brain barrier. Wilson suggested delivering the compounds intracranially, but Olson feared this would be too time-consuming. Instead, Olson suggested focusing first on substances that have been shown to be easily delivered by placing them in food or drinking water. Regardless of these optimization plans, participants agreed that, at least for now, the screening of candidates in mice should be preceded by a careful selection process.

Clinical trials

Even with a promising, animal-tested compound in hand, the path from lab-tested agent to marketed drug is long and arduous. Discussing key steps in the development of clinical trials, participants offered several suggestions on how best to overcome rate-limiting steps in the process.

To get an approximate measure of the capacity of the clinical pipeline, Tobin asked participants to estimate several of the key contributing factors. Ira Shoulson noted there are approximately 30,000 HD patients in the U.S., and 150,000 at risk. Depending on the markers used, over a period of three years, between 17 and 30% of individuals at risk aged 30 to 55 will pheno-convert --that is, begin expressing

markers of disease. Given these numbers, and the fact that the majority of HD patients are willing to enter clinical trials, so far, patients have not been a limiting factor. Referring to his experience with clinical trials testing coenzyme Q and remacemide (now in phase III), and riluzole, creatine and minocycline (now in phase II), Shoulson noted that, in fact, lotteries have been required to select participants from the large pools of volunteers. Neurologists, not patients, seem to be the limiting resource. Based on these considerations, Shoulson predicted that, in a best-case scenario, four compounds could be tested every year.

Choi was impressed by the limitations of clinical testing throughput suggested by these best-case calculations, worrisome given the large number of unknown variables and fundamentally different therapeutic approaches now on the board. He pointed out that the gulf between human HD and corresponding rodent models is likely to be larger than that extant in human stroke, where drugs effective in rodents have failed in recent clinical trials, and therefore stressed the need for developing more powerful methods of human testing, most likely based on better markers of disease progression: the "N = 1" goal of clinical testing.

Biomarkers and surrogate markers of disease

At present, HD trials are not powered to pick up small therapeutic effects. Olson noted, for example, that most of the improvements in childhood cancer came in increments of 3-10%. If these cancer drugs had been tested in trials that have the same power as the current HD studies, many of the

efficacious drugs would have been missed. As explained by Shoulson, the ability to predict pheno-conversion is key to determining the power of clinical trials. In theory, a drug that completely cured HD would require a tiny set of patients and a low level of predictability to reveal its effects. But the number of patients rises steeply when testing drugs with more moderate effects. So in order to keep patient numbers within a reasonable range, it is imperative to obtain markers that allow quantitative and reliable monitoring of the course of the disease.

Participants discussed the potential of developing several new genetic, pathological, and behavioral markers of disease. Housman, for example, noted that the identification of modifier genes might greatly enhance predictability of pheno-conversion. After repeat length, sibling age of onset seems to be the feature that is most highly correlated with the age patients of the Venezuelan group begin to develop HD symptoms. Although environmental effects could be contributing to this sibling correlation, Housman noted that the environment tends to be highly uniform across families of the Venezuelan group, strongly suggesting the existence of modifier genes. As previously mentioned, Andresen will be conducting a genome-wide search for such modifiers. A similar search may be carried out in mice as well. However, because of the difficulties involved in breeding HD mice, the human study is expected to yield results more quickly.

Several pathological markers of disease were also discussed. MRI and PET imaging were highlighted as powerful tools with the capability of

providing graded measurements of disease progression. John Mazziotta noted, however, that they are expensive procedures and the slope of the changes is shallow and non-linear when approaching the onset of disease. Monitoring surrogate markers in peripheral tissues, such as autonomic neurons and muscle, emerged as a promising alternative. Olson noted that Tom Bird, Andy Lieberman and others have demonstrated aggregates in autonomic neurons of HD patients. He suggested that the rectal ganglion and other peripherally accessible neurons be analyzed for aggregates, intracellular calcium levels, protein and gene expression changes, metabolic changes, apoptosis, dark blue cells, or other markers that might parallel degenerative changes in the brain. Shoulson agreed with the potential of biopsies, and added that olfactory tissue might also serve as a useful marker. Finally, Olson noted that NMR spectroscopy has revealed metabolic differences between muscles of HD patients and controls, providing another possible indicator of disease.

Behavioral tests may provide yet another window into the progression of HD. In a recent study, for example, researchers detected changes in subjects' ability to point accurately at targets up to seven years before the predicted onset of clinical symptoms. In addition, Graybiel suggested searching for cognitive deficits that appear early, and could correlate with disease onset.

Patient selection and funding considerations

Although patient recruitment doesn't appear to be a limiting factor, deciding which patients to include in clinical trials is still an open question. Shoulson and other participants noted that since different mechanisms are likely to

underlie onset and progression, it was important to include pre-symptomatic, in addition to symptomatic, patients in clinical studies. Certain compounds may help halt progression, yet others might be effective at preventing onset. Particularly when dealing with drugs that have proved to have no harmful side-effects, Shoulson favored conducting tests in pre-symptomatic individuals. Leslie Weiner cautioned, however, that the time and cost of setting up such trials could quickly escalate. Since mutated huntingtin is expressed even before birth, deciding when to begin treating an individual to target the initial stages of the disease could be very difficult. In addition, patients that feel healthy because they have not yet developed symptoms might be hesitant to enroll in such trials.

Regardless of the patients included, clinical trials are enormously expensive. Thus, Olson suggested that the HDF, alone or with other neurodegenerative disease foundations, lobby congress and NIH for programs similar to those developed by the National Cancer Institute (NCI). The clinical trials consortia, funded to conduct a series of trials over many years between renewals, is one such example. The Children's Cancer Group, for example, was funded \$75 million to conduct dozens of clinical trials over the course of five years. If the same model were used for HSG - or a broader consortium for all neurodegenerative diseases - investigator efforts would be focused on initiating and completing trials, rather than seeking funding for each one. In addition, the cost per trial would decrease. Olson also pointed out the NCI's rapid access to interventional developments (RAID) program, in which lead compounds identified in academia or small businesses are carried through toxicity analysis, medicinal chemistry,

and pre-clinical trials through NCI-sponsored contracts. Basically, NCI picks up the costs that are traditionally covered by large pharmaceutical companies.

Olson suspects that if these programs were coordinately pitched for HD together with other neurodegenerative diseases, the argument would be compelling. He notes that the success of the Children's Cancer Group and Pediatric Oncology Group provides an important proof of principle: overall survival for childhood cancer improved from less than 20% to greater than 75% over 25 years.

Clearly much work remains to be done towards making such headway in HD. But a wealth of encouraging signs suggests the goal of developing a cure for HD may be achieved in a perhaps not too distant future. As noted by Lemke, unlike many other neurological diseases, HD's primary cause has been pinpointed to a mutation in a single gene, providing researchers with an exceptionally clear-cut target. And even at this early stage, screening studies and experiments in animal models are already beginning to yield the first small, but tantalizing, set of therapeutic candidates.

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