

# Hereditary Disease Foundation

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## Fast Paths to a Cure

January 8-9, 2000 • Santa Monica, California  
Prepared by Marina Chicurel

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As new findings expand our knowledge of Huntington's disease (HD), a growing number of potential research paths unfold before us. The challenge now lies in selecting those that promise to yield the most benefits, most quickly. Patients suffering from the devastating symptoms of HD and those whose genes foreshadow the onset of the illness—and the potential of passing it on to their children—provide a compelling motivation to search for expeditious paths to a cure. At the same time, approaches that may prove rewarding in the long term should not be sacrificed in the name of haste. Participants at the Hereditary Disease Foundation's workshop "Fast Paths to a Cure" discussed how best to strike a balance between these goals.

Although still in the early stages of development, several compounds and procedures emerged as potential therapeutic agents. Protease inhibitors, anti-inflammatory compounds, transcriptional modulators, and regulators of energy metabolism were identified as promising candidates. And given the chronic nature of HD, gene and stem cell therapies were recognized as particularly promising options. Most participants agreed, however, that the ultimate "cure" for HD would probably involve a combination of therapies.

The extent to which understanding the mechanisms underlying HD will be required for developing effective therapies was controversial, but all agreed that basic research would be key in the long-term. Studies of huntingtin's interactions with regulators of gene expression yielded new insights into how huntingtin may disrupt cell function. And electrophysiological and molecular studies revealed early markers of disease, suggesting primary events that unleash HD's cascade of pathological changes. To extend these mechanistic studies, some participants favored focusing on unique features of HD—such as the vulnerability of the medium spiny cell—while others advocated looking for common themes—such as the similarities shared by all polyglutamine diseases.

A tone of cautious optimism pervaded the workshop. The time seems ripe for the emergence of a cure for HD, but much work lies ahead. Through stimulating discussions, participants compiled a set of strategies and tools that promise to advance the mechanistic understanding of HD, lead to the development of new therapies, and aid in the design of effective clinical trials.

## Lessons From a Family Battling HD

Members of a family afflicted by HD who shared their experiences at the workshop helped participants grasp the far-reaching and multifarious consequences of HD. Even before the symptoms of HD begin ravaging a patient and their caregivers, HD can have devastating effects on a family's life. In particular, the decision of an individual to be tested and the outcome of the test can affect each family member in many profound ways. The recent positive diagnosis of a young graduate student in her '20s, for example, her and her husband's plans

for having children. In addition, the results made her untested and pregnant sister so depressed that she stopped working, fearing the worst for her unborn child. The news also had to be broken very gently to their mother who had to face not only the knowledge of having a sick daughter, but the potentially harrowing, if misplaced, guilt of being the carrier of the mutation.

Despite the pain that accompanies a positive diagnosis, the family agreed they preferred knowing their genetic status. They felt the knowledge helped them make better life choices and deal with the problem directly. The mother of the family described having her fears dissipate to some extent when she tested positive for the disease. She had been so obsessed and fearful, watching for any hint of a symptom, that the test provided her with some relief by liberating her from the uncertainty. In addition, the family described feeling some degree of empowerment by keeping up with the latest research. Unfortunately, this information has also been a source of distress. The recently diagnosed young woman is fearful that she will experience an early onset of disease because of the large number of CAG repeats she carries.

The family's accounts also underscored the need to improve the education of physicians who deal with HD patients. The family received incorrect diagnoses, false hopes, and very little counseling when tested. In addition, they expressed a need for more information on how to obtain proper medical insurance. Although finding a cure is clearly the ultimate solution to HD, improved medical support could help ease some of the, as yet, inescapable suffering that characterizes HD.

## Therapeutic Candidates For Treating HD

### *Protease Inhibitors*

Several participants presented their encouraging first steps towards finding a cure. Protease inhibitors emerged as promising candidates for HD therapy on several counts. Since both apoptotic and non-apoptotic cell death involve protease activation, protease inhibitors could help prevent the neuronal cell loss that characterizes the late stages of HD. In a recent study (1), Robert Friedlander showed that expression of a dominant-negative caspase-1 mutant extends the survival of R6/2 mice and delays the appearance of neuronal inclusions, neurotransmitter receptor alterations, and the onset of symptoms.

Based on this work and a study showing that administration of the caspase inhibitor minocycline could reduce stroke-induced injury by 50% (2), Friedlander injected minocycline intraperitoneally into six-week old R6/2 mice and monitored its effects. His preliminary results indicate that minocycline-treated mice live 14% longer than controls, including mice injected with tetracycline—the drug from which minocycline is derived, but which does not cross the blood brain barrier. Minocycline-treated mice also showed improved performance on the rotarod, with no changes in blood glucose levels or weight. As assessed by RT-PCR of whole brain lysates, minocycline inhibited caspase-1

expression by approximately 43% and decreased caspase-3 expression by 100%, lowering it to levels indistinguishable from wildtype controls.

To tease out the effects of each caspase on lifespan, Friedlander tested DEVD, a specific inhibitor of caspase-3, Y-VAD, a specific inhibitor of caspase-1, and Z-VAD, a broad spectrum caspase inhibitor. Neither DEVD nor Y-VAD alone had significant effects on lifespan. But when administered simultaneously, they lengthened lifespan as much as minocycline or Z-VAD. Robert Smith and Gillian Bates, however, observed only a slight extension of lifespan when treating R6/2 mice with Z-VAD-FMK. Friedlander proposed that caspase-1 initiates a proteolytic cascade that triggers caspase-3 activation, which in turn leads to the unfolding of a full-fledged apoptotic program.

But there are other ways in which caspase inhibitors could exert their therapeutic effects. So far, Friedlander has only monitored expression levels, without measuring protein levels or the caspases' activities. He points out that the fluorogenic substrates used to monitor activity are probably not sensitive enough to detect small changes. But Smith and Bates have performed experiments tracking caspase activity in R6/2 mice, and they've detected only a very minor elevation in the activity of caspase 1.

The inhibitors could be acting on targets other than the apoptotic pathway. For example, they could affect production of the huntingtin toxic fragment. Smith pointed out that studies from his group have implicated caspases 3, 6, and 8, and probably caspase 1 and certain cathepsins, in the breakdown of huntingtin. He proposed that R6/2 mice may develop the cellular pathology of HD earlier than other models carrying larger constructs of mutated huntingtin because the R6/2 construct may not require processing by caspases 3 and 6. In addition, tetracycline-like compounds have been shown to affect processes beyond protease activity. They can act as calcium chelators, affect nitric oxide production, and modulate the activities of over ten different genes.

Potentially most significant, protease inhibitors can dampen the inflammation process. Dennis Choi suggested that apoptosis might be a consequence of inflammation, and that the inhibitors could be exerting their primary effect by blocking the inflammatory response. Consistent with this interpretation, Friedlander noted that minocycline has been reported to inhibit microglial activation (2). Although Bates mentioned that the occurrence of inflammation in HD brains is still subject to debate, James Olson noted that 12-week-old R6/2 mice showed increased expression of inflammatory response genes.

Since Friedlander's experiments using minocycline showed no effect on the presence of inclusions or neurotransmitter receptors, and were delayed by six weeks relative to his observations in dominant-negative mutants, Bates suggested that these experiments might involve different processes. Further studies using additional specific blockers and monitoring caspase activi-

ty—such as those developed by Smith's companies Prototek, Inc. and Enzyme Systems Products—should help clarify these uncertainties. Particularly promising are a set of inhibitors recently developed by Prototek, in which the trapping group FMK has been replaced by a different chemical trap, reducing the inhibitors' toxicity and making them better candidates for therapy.

#### *Anti-inflammatory drugs*

As suggested above, inflammation might be a prime target for HD therapy. Flint Beal described how inhibitors of COX-2, a cyclooxygenase that mediates the inflammatory response, are now in clinical trials for Alzheimer's disease (AD). Some studies have shown that AD patients have increased levels of COX-2 mRNA. Also, patients who use COX-2 inhibitors to control their rheumatoid arthritis seem to have a lower incidence of AD. And a transgenic mouse that overexpresses COX-2 appears to have an increased sensitivity to kainic acid, a neurotoxic compound.

Michael Hayden proposed looking into the databases to search for correlations between the use of anti-inflammatory drugs for arthritis, for example, and a decreased prevalence of HD. Since the COX-2 inhibitors are FDA-approved and already being used in other neurodegenerative disease trials, Nancy Wexler wondered if they might be good candidates for testing in the near future. However, Anne Young thought the data were still too scarce to warrant recruiting the large number of subjects required to run a solid clinical trial.

William Richards suggested testing other anti-inflammatory agents, such as the new peroxisome proliferator-activated receptor (PPAR) agonists which, as shown by the work of Gary Landreth (3), are capable of inhibiting microglial activation in models of AD.

#### *Transcriptional Regulators*

##### **Glucocorticoids**

Two novel therapeutic candidates—glucocorticoids and retinoids—were proposed based on their ability to regulate the transcription of specific sets of genes. Marc Diamond described how glucocorticoids can regulate aggregation and nuclear localization of expanded polyglutamine polypeptides derived from the androgen receptor and huntingtin through specific regulation of gene expression (4). Using the HEK 293 human kidney cell line and the N2a mouse neuroblastoma cell line, Diamond found that the wild-type glucocorticoid receptor suppressed the aggregation and nuclear localization of these proteins. Mutations within the DNA binding domain and the N-terminus transcriptional enhancer domain of the glucocorticoid receptor abolished the activity. On the other hand, deletion of the transcriptional enhancer domain increased aggregation and nuclear localization two to three times over wildtype controls. Diamond thinks the deletion construct may act as a dominant-negative mutation, suppressing the endogenous receptor's activi-

ity. Thus, cellular processes that can be modulated by glucocorticoid receptors may provide a powerful handle for regulating the aggregation and nuclear localization of expanded polyglutamine proteins.

To test this concept *in vivo*, Diamond plans to use implanted pellets to deliver dexamethasone into the brains of R6/2 mice. Because the modulation of aggregation and localization require high levels of glucocorticoid receptors, he also plans to make transgenic mice that overexpress the receptor. Beal cautioned that steroids exacerbate diabetes, such that he should use either Hayden's mice or very young R6/2 mice, before they develop diabetic symptoms. Finally, Diamond hopes to use DNA microarrays to identify the glucocorticoid-regulated genes involved in modulating aggregation and localization. And since Olson is interested in examining the effects of anti-inflammatory compounds on gene expression patterns, the two may collaborate on the project. Although finding these genes would likely provide important new therapeutic options, Richards cautioned that, from a drug development perspective, finding agonists is usually much more challenging than finding antagonists. Thus, he favored developing additional screens to identify dominant acting enhancers of recessive suppressors.

The glucocorticoid receptor may exert its effects in several ways, including the suppression of inflammation (see above) and/or the activation of a stress response. Allan Tobin wondered if stress might thus be beneficial for HD patients. Some participants reported that stress, such as handling or receiving injections, seemed to make R6/2 mice live longer, yet others reported the opposite. Olson noted that, using gene chips to monitor gene expression patterns, his group found that the striata of 12-week-old R6/2 mice express increased levels of several stress-associated genes. He also speculated that the reason why many HD patients seem to first notice their HD symptoms after being sick or injured could be because glucocorticoid levels drop after the stress from an illness or accident subsides.

#### *Retinoids (Vitamin A derivatives)*

From his gene chip studies, Olson also obtained data suggesting that impaired retinoid signaling might contribute to HD pathology. Retinoids induce the transcription of genes by binding to nuclear hormone receptors. Through these receptors, they regulate several processes—including neural differentiation. Olson's results indicate 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 strongly suggest that retinoids may prove effective for treating HD. To test this proposal,

Olson has begun administering retinoic acid to R6/2 mice. In a preliminary experiment, he found that the development of HD symptoms, as assessed by a foot-clasp reflex assay, was delayed by approximately one and a half weeks in R6/2 mice that received retinoic acid at six weeks of age. In addition, Olson mentioned that Pierre Chambon has described a retinoid receptor knock-out mouse with an HD-like phenotype. Richards suggested Olson define the retinoid target by introducing retinoid receptors using viral or retroviral vectors to attempt rescuing the HD phenotype. Robert Darnell added that testing various doses of RXR by introducing constructs with variable copies of the gene might also be useful. Although encouraged by these preliminary results, Olson noted that finding an optimal dose of retinoic acid with minimum side-effects might be challenging since retinoids can be toxic when administered in large doses.

### **Regulating Huntingtin Expression**

Regulating the expression of huntingtin itself emerged as another approach warranting further investigation. Hayden's group observed that wildtype huntingtin plays a key role in modulating the effects of mutant huntingtin. Crossing YAC72 mice (containing huntingtin with 72 CAG repeats—see ref. 7) with huntingtin knockout mice, they found that the offspring's testes—used as surrogate markers of disease—suffered severe atrophy with massive apoptotic cell death. Heterozygous YAC72 mice, on the other hand, seemed to fare much better. Although the knockout and YAC72 mice did not belong to the same strain, Hayden's results suggest that wildtype huntingtin somehow tempers the deleterious effects of the mutant. Thus, upregulating the expression of wildtype huntingtin could potentially offer therapeutic benefits.

### **Compounds to Treat Mitochondrial Dysfunction and Oxidative Damage**

Several neurodegenerative diseases, including HD, seem to be characterized by mitochondrial dysfunction and oxidative damage. Hoping to counteract this pathology, Beal tested the effects of compounds that affect energy metabolism on the survival of R6/2 mice. Several agents, such as coenzyme Q10 and dichloroacetate, seemed to enhance survival by approximately 10%. More promising results, however, were obtained with creatine. When administered as 2% of the animals' food volume, creatine increased survival by 17.5% in R6/2 mice, and by approximately 15% in David Borchelt's mice. Doses below and above 2% enhanced survival to a lesser degree. Rotarod performance, body weight, and brain weight paralleled the enhanced survival. In addition, brain atrophy and striatal nuclear inclusions appeared reduced 30, 60, and 90 days after beginning the administration of 2% creatine.

It is yet unclear how creatine mediates these neuroprotective effects. On the one hand, creatine boosts production of phosphocreatine, which serves as a buffer against energy loss.

As such, it help keeps the sodium-potassium ATPase running and fuels the reuptake of glutamate. On the other hand, creatine may also be involved in regulating the mitochondrial permeability transition associated with cell death, since creatine kinase seems to participate in mitochondrial pore formation.

Beal plans to extend his studies by dissecting creatine's mode of action and assessing creatine concentrations in the brains of treated mice through the use of imaging techniques. In addition, Friedlander is beginning to use cell-free systems to examine how creatine inhibits cell death. And since creatine appears to mediate neuroprotection through a pathway different from that mediated by the caspase-1 dominant negative mutant and the ZVAD inhibitor, he has begun experiments combining creatine and caspase inhibition. Friedlander also suggested initiating studies combining minocycline with creatine.

Establishing a dose that is safe for human trials is another important goal. Beal noted that five to ten grams a day seemed safe in the short-term, but its long-term effects remained untested. Based on Ai Yamamoto's results showing that formation of huntingtin inclusions can be reversible, Choi proposed testing creatine for its ability to eliminate pre-formed aggregates.

### **Beyond Drugs: Potentially Longer Term Solutions**

As pointed out by Friedlander, developing therapies for chronic diseases can be particularly challenging because the therapies must retain their efficacy over a lifetime. Gene therapy and stem cell therapy surfaced as promising candidates to circumvent this problem.

#### *Gene therapy*

As described by Ron Mandel, recent advances in gene therapy have transformed it into a highly powerful and promising technique for both therapeutic and research purposes. For example, he noted that, using a Lenti virus in rats, he can permanently and reliably transduce 40 to 50% of all striatal neurons.

Mandel proposed using gene therapy to introduce genes with potentially neuroprotective activities into the brains of HD mice. One of his first goals, for example, is to introduce a ribozyme to knock-out huntingtin expression. He is also planning to introduce genes encoding trophic factors, such as CNTF and BDNF, using an adeno-associated virus (AAV). Even though AAV is not as efficient as the Lenti virus, he calculates he'll only need a 5% transduction efficiency to completely bathe the striatum with the growth factors. Hayden pointed out that two other groups are also introducing trophic factor genes into wildtype mice and YAC models of HD.

In addition to trophic factors, genes such as caspase inhibitors and modulators of neurotransmission, may also serve as therapeutic agents. Evan Snyder noted that genes that encode secreted factors or peptides are relatively easy to deliver and

could serve as constant "drug" sources to deal with HD's chronic nature.

Participants discussed several technical strategies to get the most out of gene therapy. Mandel stressed the need for regulatable vectors that can be switched off if necessary, especially when introducing genes that modulate neurotransmission. Ethan Signer proposed using bidirectional promoters when attempting to introduce two genes simultaneously. Because AAV is limited in the size of the DNA fragments it can carry, inserting two genes often requires packaging each gene into a separate vector, reducing transduction efficiencies substantially. And to obtain a greater distribution of introduced vectors, Snyder suggested using stem cells as packaging/producer lines. A large number of vectors could be delivered to extensive areas of the brain using only small numbers of stem cells.

#### *Cell therapies*

Beyond their potential as vector delivering agents, stem cells are promising therapeutic agents per se. Snyder described how placing stem cells in the brains of nervous mice mutants, which lack most of their Purkinje cells, resulted in the restoration of the cerebellar cells. Interestingly, the same stem cells failed to produce Purkinje cells in adult wildtype mice. Snyder speculates that the landscape of the injured brain may allow stem cell differentiation because it shares important similarities with the developing brain. This observation may be particularly pertinent for HD, since several researchers have noticed similarities between HD brains and developing brains (5). For example, medium spiny cells have small numbers of dendritic spines and lack inward rectifying currents during early development as well as in mouse models of HD. In addition, during normal development, animals experience a physiological chorea reminiscent of HD chorea.

Stem cells also seem to owe their potential of rebuilding injured areas to the fact that inflammation factors attract stem cells. If inflammation is occurring in HD as discussed above, stem cell therapy might be particularly effective. Of course, in this case it may be important to avoid blocking inflammation completely.

### **Candidate Therapies: Concluding Remarks**

Several participants predicted that, rather than a single therapy, a combination of therapies would ultimately provide the hard-sought cure for HD. As pointed out by Mandel, the encouraging but modest effects of the candidates tested so far support this view. Beal cited cancer and AIDS as examples of diseases that are being controlled by this kind of approach. In addition to developing combination therapies, searching for drugs and targets that affect multiple aspects of HD might be particularly rewarding. Marie-Francoise Chesselet cited birth control pills as an example of a drug that works extremely well because it acts at many different levels to prevent conception.

Based on this idea, Chesselet stressed the need to screen drugs by monitoring endpoint markers of disease in order to assess their cumulative effects. Young noted an even more fundamental reason to expect success from therapies that attack HD at several levels—the likely multiplicity of mechanisms by which mutated huntingtin causes disease.

### **Mechanisms Underlying HD**

Participants differed in their views regarding the extent to which mechanistic knowledge should back the development of new therapies. John Marler reminded participants that the mechanisms underlying the action of most drugs on the market are not well understood. And Signer pointed out that just as searching for mechanisms can lead to the development of new therapies, searching for therapies can lead to mechanistic understanding. He supported the use of random drug screens, in part, because of their potential as mechanistic probes. Given the potential of systems such as yeast, cell cultures, and *Drosophila* to screen large amounts of compounds quickly, Olson also supported drug-searching efforts that are not based on mechanism. He added that it might be wise for the Hereditary Disease Foundation to support random screens since the pharmaceutical industry is unlikely to do so.

On the other hand, Richards advocated for the search of validated targets. If partnering with industry is one of the foundation's goals, then finding these targets will be essential. In the case of caspase-1 inhibitors, for example, Richards noted the effect seen in mice was probably not enough to motivate companies to initiate clinical trials. If its mechanism of action were identified, however, it could lead to the development of more efficacious drugs. Richards acknowledged that mechanistic knowledge is lacking for many modern drugs, but he feared that performing random screens would probably generate numerous hits, and following each into leads could turn out to be very time-consuming. Diamond agreed that research focused on mechanistic problems is essential. He felt that the cell biology of polyglutamine expansion disease was given short shrift at the meeting, and stressed the need to probe issues such as the distinct roles of the nucleus and cytoplasm in the pathology of disease, and the roles of soluble proteins versus micro- and macro-aggregates.

But beyond their differences in opinion regarding fast-track approaches, most participants agreed that basic mechanistic research would be key in the long-term. Attesting to its unequivocal importance, several of the mechanistic results presented triggered new ideas for therapeutic intervention.

### **Huntingtin as a Disruptor of Transcriptional Regulation**

Leslie Thompson presented a compelling new model for how huntingtin could mediate cell loss. Using glutathione-S-transferase (GST)-fusion proteins and co-immunoprecipitation techniques, Thompson's lab (work done by Joan Steffan) dis-

covered that huntingtin exon 1 interacts with two transcriptional regulators: p53 and the transcriptional co-activator CREB binding protein (CBP)—a transcription factor involved in apoptosis that interacts with p53. Exon 1 containing the proline-rich domain of huntingtin, which is very similar to that of p53, binds to p53 in a manner dependent upon the C-terminus of p53 (which contains the tetramerization domain). Both wildtype and mutant huntingtin bind to p53, but only the mutant represses the transcription of genes normally regulated by p53. In the case of CBP, its interaction with huntingtin is dependent upon the length of the repeat in huntingtin, the presence of the polyQ repeat in CBP and the presence of the prolines in huntingtin.

Thompson speculates that mutant huntingtin causes transcriptional disruption by mimicking or modulating the function of p53 and/or sequestering CBP, ultimately leading to neuronal cell death. Beal noted that the binding could occur either in the cytoplasm or in the nucleus. Consistent with the possibility of CBP sequestration, Bates has found CBP in the inclusions of R6/2 mice. Even more suggestive, a recent study showed that HD patients seem to have a reduced incidence of cancer (6). If mutant huntingtin is acting like p53, one would expect it to suppress tumor formation.

Thompson's model suggests a possible explanation for cell loss not only in HD, but in the other polyglutamine diseases since all polyglutamine proteins contain proline-rich regions adjacent to the polyQ repeats, containing at least one PXXP domain (consensus SH3 domain interaction site). The model has yet to explain, however, the specific vulnerability of different cell types that characterizes these diseases. Thompson pointed out that neuronal apoptosis is unique in several aspects and that this might explain the neuronal, if not brain region, specificity. Potentially other brain region specific proteins are involved in these processes as well.

To extend her studies, Thompson plans to test full-length huntingtin and perform co-immunoprecipitations using brain tissue, in addition to continuing the mechanistic studies in the over-expressing cell lines she has been using so far. Darnell suggested using epitope-tagged proteins. To get an idea of the specificity of the interactions, Tobin suggested assessing the total number of proteins that co-immunoprecipitate with huntingtin. Hayden noted that yeast two-hybrid screens have identified approximately 10 proteins that seem to interact with huntingtin. Possibly because CBP and p53 are transcription factors they had not been previously identified, although the transcription factor N-CoR was identified as an interactor by a yeast two hybrid screen (Lesley Jones).

### **Understanding HD Progression**

One of the most pressing issues in HD research today is distinguishing primary from secondary effects. A growing list of HD-associated abnormalities is being compiled, yet little is

known about how these pathologies are linked to each other through cause and effect. Bates noted that much of HD research has been based on observations of brains from HD patients in the late stages of the disease and that there is very little information on sequential events. She and others strongly favored the search for early events.

Perhaps the approach that has yielded the most sequential data, and which promises to most clearly elucidate the natural history of HD at an anatomical level, is the use of imaging technologies. John Mazziotta described how structural imaging has revealed the relentless atrophy of the striatum that proceeds in a dorsomedial to ventrolateral direction. In later stages, atrophy of the frontal cortex is observed. Closely paralleling these observations, functional studies indicate an initial reduction in caudate and pallidal function, accompanied by increased activity in the thalamus, due to its disinhibition. As the disease progresses, however, activity in the posterior thalamus declines as well. Citing Elizabeth Elwood's work using MRI and PET imaging, Hayden noted that this serial decline follows a highly predictable time course that can be tracked even in pre-symptomatic patients.

Although imaging techniques are extremely useful for monitoring the disease at the whole brain level, additional approaches are required for identifying the molecular mechanisms underlying HD. Olson has used gene chips to tackle this problem. As described above, he monitored the gene expression profiles of six- and 12-week old R6/2 mice. Of the nearly 6000 mRNAs he measured, a small subset—mostly comprising mRNAs encoding neurotransmitter, calcium, and retinoid signaling pathway components—were decreased. This approach's potential for dissecting early events prompted several participants to support the organization of a future workshop on DNA microarrays and gene chips.

Hayden presented evidence for one of the earliest pathological events discovered to date. Working with Lynn Raymond, Hayden transiently co-expressed NMDA receptors NR-2A and NR-2B with mutant huntingtin in HEK cells. Cells expressing NR-2B, but not NR-2A, showed a hypersensitivity to glutamate with increased apoptotic cell death. Based on this data, Hayden proposed that increased glutamate sensitivity might be a feature that is expressed very early and that triggers the activation of multiple protease pathways, particularly caspase-3. The early increase in neuronal calcium levels he has observed in YAC72 mice is consistent with this hypothesis.

Two previous studies on glutamate sensitivity, however, yielded somewhat contradictory results. Patrik Brundin found that R6/2 mice were actually more resistant to quinolinic acid excitotoxicity in the striatum than wildtype controls (8). However, Hayden stressed that the glutamate hypersensitivity he observes requires full-length huntingtin. In addition, timing might be critical. Glutamate sensitivity might change during disease progression. In fact, Brundin suggests that his observations could

be the result of striatal cells having recruited increased defenses against excitotoxic death after having been primed by a sub-lethal dose of glutamate.

The second study, carried out by Levine (9), yielded similar results to Hayden's with two exceptions, as pointed out by Chesselet: Levine observed hypersensitivity using both truncated and full-length huntingtin, and recorded an approximately 10 mV depolarization in HD cells relative to controls. Hayden noted that, although he didn't observe a change in membrane potential, if a change occurred that was mostly restricted to the cells' dendrites, he would've been unable to detect it. Strain differences could also account for some of the differences in their data.

To extend Hayden's results, James Surmeier suggested teasing apart the mechanism underlying increased glutamate sensitivity. He proposed designing experiments to discern whether depolarization, increased receptor expression, or allosteric changes in receptor structure, for example, could be pinned down as the culprits. And to further explore the link between huntingtin and NR-2B receptors, Young suggested crossing HD mice with NR-2B knockout mice, as well as studying the effects of NR-2B antagonists on the cells of HD mice.

## **Putting it Together**

Young assembled a particularly compelling model of how HD disrupts cell function by integrating several of the mechanistic clues provided by other participants. She proposed that HD pathology is the result of a cascade of multiple events that ultimately snowballs out of control. Dysfunction begins when mutant huntingtin causes an increase in glutamate sensitivity which then leads to caspase activation. The activated caspases cleave huntingtin generating fragments, some of which slip into the nucleus. These fragments interfere with transcription by binding to factors such as CBP and p53, which then cause cellular stress. The stress leads to more huntingtin cleavage and a vicious cycle ensues, accelerating until the cell dies.

In addition to its ability to explain several of the disparate results presented at the workshop, Young's model suggests a therapeutic approach to HD. The model supports the development of distinct therapies to attack the various stages of the disease. For example, in the early stages, it might be best to treat glutamate toxicity or attempt to buffer calcium. As the disease progresses, however, one could turn to more aggressive therapies, such as the administration of protease inhibitors.

## **Future Strategies and Approaches**

*Basic research: A search for peculiarities or commonalities?*

When trying to dissect the mechanisms underlying HD pathology, one can focus on its unique features or search for common themes. For example, one can try understanding the particular vulnerability of striatal cells, or ask why neurons, in general, seem to be affected by HD. At another level, one can try understanding the specific functions of huntingtin, or instead search for mechanisms shared by the various polygluta-

mine diseases. Although most participants agreed that both approaches were necessary, they varied widely in the importance they assigned to each.

#### *Is HD a disease of the enkephalinergic medium spiny neuron of the striatum?*

The striking susceptibility of the medium spiny cells of the striatum to the effects of mutant huntingtin has long intrigued researchers. To test whether the spiny cell's cellular milieu is responsible for this exaggerated vulnerability, Beal proposed targeting mutant huntingtin to other cell types through the use of cell type-specific promoters such as the tyrosine hydroxylase promoter. He noted that similar experiments in ALS mice have shown that targeting the gene to astrocytes alone, for example, is not enough to cause disease.

To elucidate the molecular underpinnings of the spiny cell's sensitivity, Surmeier suggested probing the functions and subcellular localization of its unique set of ion channels and neurotransmitter receptors (5). Such studies may reveal the mechanisms underlying the elevation in membrane potential, disruption in calcium dynamics, and altered responses to dopamine and glutamate observed in HD spiny cells. Surmeier suggested, for example, that the enkephalinergic spiny cells' set of Kir potassium channels might explain the altered membrane potential described by Levine (9).

Hayden's experiments on glutamate sensitivity also offer clues on HD's apparent cell specificity. Although NR-2B receptors are not restricted to the spiny enkephalinergic cells of the striatum, they are highly expressed in these cells. In addition, Hayden noted that the cellular and subcellular localization of caspases is very variable among different cell types, which could result in differences in apoptosis and huntingtin cleavage. Tobin encouraged Surmeier and Hayden to work together at dissecting these early molecular events.

#### *Or is HD a general disease of neurons?*

But it is still uncertain whether cells in the striatum are intrinsically vulnerable to mutant huntingtin or suffer from the consequences of disrupted cortical inputs. Mandell pointed out that quinolinic acid's toxic effect on striatal cells decreases greatly when the frontal cortex is removed. And Chesselet described how she and Levine observed, using Golgi and biocytin fills, that the spiny neurons of HD mice tend to lose their dendritic spines, presumably losing their cortical input. Furthermore, Young has detected a downregulation in the expression of metabotropic Glu-R2 glutamate receptors in the striata of HD mice that are only four weeks old.

Some studies suggest that beyond striatal vulnerability, HD may be a global disease of neurons. As Bates pointed out, citing Jack Penny, HD seems to be a multi-system disorder given that the decrease in brain weight observed in HD patients is much greater than the total weight of the striatum. Furthermore, both

Hayden and Daniel Madison (10) have recently found impairment of LTP in the hippocampi of HD mice models. And as noted by Chesselet, patients with large numbers of CAG repeats suffer from neuronal atrophy in many brain regions in addition to the striatum and cortex. A new hypothesis offering an explanation for neuronal susceptibility to mutant huntingtin was pointed out by Signer. In a recent paper, John Trowsdale and colleagues showed that huntingtin and ataxin-1 inclusions disperse during mitosis, reducing the concentration of aggregates in the nucleus (11). They thus proposed that, because neurons are post-mitotic cells, they may be particularly vulnerable to the presence of nuclear inclusions.

#### *HD: Unique or similar to other polyglutamine diseases?*

In addition to offering an explanation for neuronal vulnerability, Trowsdale's study highlights a striking similarity shared by the polyglutamine diseases in the formation of nuclear and cytoplasmic aggregates. Based on this common pathology and the fact that the localization and formation of both HD and spinobulbar muscular atrophy aggregates are similarly regulated by glucocorticoids, Diamond urged participants to search for common mechanisms shared by these diseases. As previously mentioned, Thompson's model also supports a common mechanism of disease, since all polyglutamine proteins contain similar proline-rich regions.

But Darnell cautioned that trying to generalize across diseases could be misleading because the primary pathologies are still unknown. Early events, such as the glutamate hypersensitivity described by Hayden, may be the key triggers of disease, and there is no evidence yet to suggest these are shared mechanisms.

Searching for a possible indicator of shared mechanisms, Tobin asked if, in their early stages, all polyglutamine diseases caused similar clinical symptoms. At least in his mouse models, Borchelt said, each disease was phenotypically distinguishable early on—with the possible exception of DRPLA and HD. Several participants noted, however, that comparing clinical manifestations was difficult because of their extreme variability. According to Diamond, for example, even individuals within the same family can express the same disease very differently.

Hayden noted that, except for ataxin-1, cleavage of polyglutamine proteins shared similar themes but differed in the proteases involved. A comparison of gene expression profiles by DNA chip analysis, as suggested by Mandel, may ultimately help determine the true extent of the similarities shared by these diseases and provide insights into their fundamental mechanisms.

#### *Methodological strategies*

A wealth of methodological strategies to resolve mechanistic questions and develop effective therapies was proposed by workshop participants. As Signer and Hayden pointed out, the challenge will lie in integrating them—bridging the gap between such distinct approaches as molecular biology and electrophysiology.

At a more utilitarian level, several participants called for a standardization of protocols and reagents, including equipment for studying behavior, brain dissection techniques, DNA chip analyses, and mice strains. Borchelt, for example, described the variability in disease onset that can be introduced by factors such as litter size and a mother's genetic background. To establish guidelines, Tobin proposed setting up a workshop to deal exclusively with these issues. In addition, Hayden and Borchelt suggested creating core facilities for making technologies, such as DNA chips, widely available.

#### *Pharmacological approaches*

Several participants favored a pharmacological approach as the first line of attack against HD. As one of its more enthusiastic proponents, Choi noted that pharmacological experiments could provide a panoramic view of the landscape of HD in a relatively short time. He also stressed the importance of applying the lessons learned from well-characterized diseases to HD research. For example, stroke research revealed that injury involves multiple pathways and that moving from animal models to humans is often less straightforward than expected. Drugs that seemed very effective in rat models of stroke, such as NMDA receptor antagonists, ultimately failed in human clinical trials. Thus, he cautioned against focusing too narrowly on specific molecular targets. Instead, Choi suggested starting out by testing a wide range of drugs for their neuroprotective capabilities. And to obtain quick estimates of their effectiveness, doses, and bioavailability, he suggested using stroke as a model system.

A wealth of parameters, ranging from survival to molecular changes, were discussed as candidates for monitoring drug effectiveness. Chesselet argued that it was important to use end-point markers of disease to track a drug's cumulative effects, especially since the most promising drugs are likely to have effects at multiple levels. Choi agreed and suggested tracking the presence of inclusions or cell death.

But others favored using earlier markers to track changes with a defined mechanism. Signer and Richards, for example, suggested monitoring calcium levels based on Hayden's results showing increased calcium in the hippocampal neurons of 6- to 10-month old YAC72 mice. Richards noted that the rise in calcium could be closely linked to cytotoxicity and that calcium screens are robust and relatively easy to perform. But since the role of calcium in HD is yet to be established, Choi considered it was premature to select it as a marker. Regardless of the selected marker, however, several participants agreed that pharmacological compounds that have already been approved by the FDA should be given priority.

Not everyone embraced the pharmacological approach, however. Surmeier, for example, cautioned that pharmacology could be misleading due to the lack of specific agonists and antagonists for molecules such as ion channels.

#### *Genetic approaches*

Darnell championed a genetic approach to sort out the roles of the multiple targets that seem to be involved in HD. In particular, he suggested using a systematic knockout approach. Although Diamond and Beal thought that creating knockouts would be too time-consuming, Darnell noted that several of the required knockouts, such as the caspase-1 knockout, are already available. He considered that within a year, many of the major targets could be ruled in or ruled out through the use of knockouts. Signer cautioned, however, that knockouts can fail to yield information when a gene's functions overlap with those of other genes.

Gene therapy emerged as another approach to tackle HD. In addition to its potential as a therapeutic agent, gene therapy allows for the introduction of ribozymes, antisense, marker genes, and the over-expression of endogenous genes in a tissue-specific manner, as noted by Mandel. And because neurons can now be transduced specifically and permanently, it appears well suited for the study of brain diseases such as HD.

As previously mentioned, support for the use of DNA microarrays surfaced repeatedly. Their potential for revealing early pathological changes promises to greatly further the understanding of disease mechanisms and aid in the selection of targets for treatment.

And although most consider the genetics of human HD a resolved issue, Borchelt reminded participants of the potential importance of modifier loci. Identifying additional genes that influence the expression of HD could offer both mechanistic and therapeutic insights.

#### *Cellular approaches*

Before engaging in genetic studies, Surmeier favored probing the cellular and molecular basis of HD to pinpoint targets more precisely. He thought that electrophysiological studies at a cellular level (5) could be carried out relatively quickly, setting the foundations for other approaches.

Other participants suggested focusing on huntingtin and aggregate formation. As proposed by Signer, one way of looking at the task of finding a cure for HD is finding a way of making polyglutamine repeats invisible to cells. Richards proposed continuing studies on the heat shock response and protein folding. Young and Beal stressed the importance of dissecting the distinct roles of nuclear versus cytoplasmic inclusions.

Stem cells surfaced as a powerful tool to complement classic cell and molecular biology approaches. Snyder explained how stem cells derived from animals with a genetic background that differs from that of their hosts can be used to create chimeras that reveal the physiological effects of specific genes or mutations. In the case of HD, for example, stem cells from a wild type mouse could be injected into the brains of HD mice to produce chimeras expressing mutant huntingtin everywhere except in their striata. Such mice could help elucidate whether

striatal atrophy is caused by the direct effects of mutant huntingtin on striatal cells or by its effects on the striatum's cortical inputs, as mentioned above. They could also help reveal to what extent striatal atrophy is responsible for HD symptoms. A genetic marker, such as the gene encoding green fluorescent protein (GFP), could be inserted into the stem cells, before implantation, to monitor their behavior in the host. Thus, stem cells could be used not only to replace damaged cells and deliver genes for therapeutic purposes, but as reporters of the disease process.

#### *Animal models*

Using amyotrophic lateral sclerosis as an example of the power of animal models, Borchelt stressed the need for producing better models of HD. Several models were identified as candidates for accelerating the screening of drugs. Hayden described his efforts developing mouse YAC models that express the HD phenotype earlier than his original YAC mice. He is creating mice with higher levels of mutant huntingtin expression and with longer CAG repeats—including a new line carrying 128 repeats. The new mice promise to facilitate drug screening, as well as natural history research. The creation of a full-length, inducible mouse by Borchelt should also shed light on disease progression. The mouse he has created, carries a TET-off inducible mutant gene with 148 CAG repeats and a myc-tag. When the gene is turned on, the mouse exhibits a profoundly abnormal gait. Thompson noted that given Friedlander's results with minocycline, the potential side-effects of tetracycline on proteases should be monitored.

Simple systems, such as *Drosophila*, emerged as promising models for high throughput screening of drugs. As described by Thompson, flies are cheap to maintain, reproduce quickly, are genetically well characterized, and lack a blood brain barrier. She has generated mutants carrying huntingtin exon 1 and mutants carrying an inducible gene coding for a peptide with polyglutamine repeats which is expressed in all neurons. When the inducible gene is turned on, the flies' offspring die in the larval stage, in a temperature-dependent manner (due to increased expression with increased temperature). Thompson hopes to use these flies to screen candidate drugs by placing the drugs directly in the flies' food. Choi pointed out the importance of controlling for food consumption, since it could be a significant source of variability. It will also be important to set up an efficient system for handling the flies. Thompson considered Richards's suggestion of using a system analogous to 96-well plates a feasible option.

#### *Imaging technologies*

Several participants classified neuroimaging as a key technology for gaining a deeper understanding of HD's natural history and developing new therapies. The non-invasive nature of imaging and its ability to detect presymptomatic changes make it a powerful tool to track the outcomes of clinical trials. Mazziotta predicted that the efficiency, sensitivity, and power of

clinical trials would be greatly improved through imaging technology. He also thought that several imaging groups might be interested in studying HD since it is relatively well-described, accurately diagnosed, can be modeled in animals, and is amenable to multi-variate analysis. Through collaborations with these groups, researchers could follow patients grouped by different parameters, such as CAG repeat lengths, and extract correlations from large sets of data obtained using functional MRI and PET imaging. As mentioned below, the Predict-HD (Neurobiologic Predictors of Huntingtons Disease Onset) trial is beginning to undertake such kinds of studies.

Mazziotta has been recently funded to create population/probability atlases that will compile imaging information from very large numbers of normal animals in four dimensions, spanning space and time. He urged participants to suggest stains and tests to include in his study, since they could serve to establish baseline measurements for future studies of disease.

#### **Clinical trials**

Even with a promising candidate in hand, the path from lab-tested agent to marketed drug is often long and arduous. Participants discussed how best to standardize and optimize clinical procedures, and how to avoid common pitfalls that have hindered drug development in the past.

#### *Choosing Candidates For Clinical Trials*

Olson proposed setting up a set of standards or goals as minimum requirements for candidate therapies to be considered for clinical trials. He feared that without such guidelines, researchers might try rushing their chosen candidates into trials prematurely. As an example of such guidelines, Olson suggested requiring data from at least two different animal models, and at least two independent labs. Chesselet added that each candidate should be tested in both truncated and full-length models of HD.

But Marler cautioned that establishing too many rules at such an early stage could eliminate candidates that might ultimately prove efficacious. And Young noted that such guidelines can become "moving targets" because of the continuous development of new reagents and technologies. In Marler's view, the combination of several factors is what should determine whether a candidate therapy is approved for clinical trials or not. For example, a drug with a very low risk of causing adverse side effects could be worth considering, even if its effects on animal models are only modest. And although helpful, Marler thought mechanisms of action were not indispensable—the underlying mechanisms of most drugs remain unknown. In contrast, he considered that establishing dosage and timing effects was part of the critical preparation required for entering clinical trials. Finally, he noted that clinical trials are often based on controversy. Interestingly, therapies that have both strong supporters and strong opponents usually yield the best results.

### *Designing Effective Clinical Trials*

Mandel suggested defining unifying clinical procedures as “gold standards” for carrying out the clinical trials. He described how placebo effects, highly variable outcome measures, and the lack of FDA oversight, for example, rendered many of the Parkinson’s disease (PD) trials uninterpretable. A standardized protocol—the Core Assessment Program for Intracerebral Transplantations (12)—was eventually agreed upon by the PD research community, but only after much time and effort had been lost. Establishing standards early on, he added, would also help identify points of comparison between animal models and clinical trials. Hayden favored developing partnerships with pharmaceutical and biotechnology companies since, as academic researchers, most participants were probably relatively naïve about drug development. And Friedlander suggested communicating regularly with the HSG so that both groups could benefit from each other’s advances.

A potentially complicating factor brought up by Choi was the risk that the natural history of HD is changing with time. Many patients with HD are now beginning to take vitamin E and coenzyme Q10, and in the future they may begin taking other compounds as they try keeping up with the latest research developments. Recognizing this fact and its potential to undermine future clinical studies, Olson encouraged the identification of a “best available therapy” (BAT) to replace placebo controls. He noted that many patients in previous HD trials took supplemental, non-study drugs because they feared they were receiving the placebo treatment. To minimize this problem, Olson suggested the HDF develop a consensus BAT described as “a therapy that is believed by a number of HD clinicians to be beneficial to HD patients and which has minimal documented toxicity.” In addition to providing a more acceptable arm than conventional placebo for patient enrollment, it could help reduce the number of patients taking non-study supplements.

Given the effort and cost involved in conducting clinical trials, some participants suggested ways to minimize the number of patients per trial. Olson suggested conducting multi-armed trials to reduce the numbers of patients on placebo. This approach would not only reduce trial costs, but make it more appealing to patients. Similarly, Borchelt suggested “piggy-backing” on clinical trials for other diseases, such as AD. Young noted, however, that finding age-matched controls might be difficult since AD patients are, on average, much older than HD patients.

### **Measuring Therapeutic Effects**

As explained by Hayden and Young, one of the key problems previous clinical trials have suffered from is being underpowered due to a lack of solid baseline measurements. In a trial testing the effects of Lamotrigene, for example, researchers had a 10% likelihood of detecting a 50% effect! Two new trials organized by the Huntington Study Group (HSG) are beginning to address this problem. Pharos (Pilot Huntingtons

Disease At-Risk Observational Study) is a prospective double-blinded study that will determine pheno-conversion rates by monitoring people who are at risk, but do not know their genetic status. Predict-HD (Neurobiologic Predictors of Huntingtons Disease Onset) is a study of neuropsychological, behavioral, MRI and PET parameters in pre-symptomatic patients. Both studies promise to provide crucial data on the natural history of HD that should allow future clinical trials to yield significant results more rapidly, using less patients.

The Pharos and Predict-HD studies rely heavily on the choice of reliable biomarkers. Although most participants labeled imaging techniques as excellent markers, many advocated for the search of additional surrogate markers in humans and mice. Pointing out their importance in both clinical trials and drug development, Carl Ferry labeled the search a top priority. Hayden described how his lab is using testes as surrogate markers in mice. And Olson suggested monitoring the nerve plexuses of rectal tissue in humans, emphasizing that rectal biopsies can be easily obtained.

Two recent studies suggest new behavioral markers for tracking HD progression. In one study, researchers detected changes in subjects’ ability to point accurately at targets up to seven years before the predicted onset of clinical symptoms (13). In the other, researchers proposed bradykinesia as a reliable predictor of HD progression which correlates with the loss of D2 binding in the striatum (14).

To identify additional early markers, Chesselet suggested a clever approach used by researchers studying schizophrenia. In blind studies, the researchers observed old videotapes of the patient’s birthday parties, before they had developed clinical symptoms. Searching for subtle differences in behavior, they then tried predicting which children would later develop the disease.

### **Concluding Remarks**

Although as pointed out by Bates, only a small sample of the whole of HD research was discussed at the workshop, many new insights emerged that promise to help move the HD research community one step closer to finding a cure. To accelerate the search, several participants proposed survey approaches, such as high through-put screening of large numbers of drugs. Others favored honing in on the primary causes of HD using electrophysiology, cell, and molecular biology approaches to identify validated targets. Ultimately, as Chesselet pointed out, the HD research community can’t afford not pursuing both.

Standardization emerged as a recurring theme. From mice strains to rotarods, participants called for setting up uniform guidelines for protocols and reagents. In the design of clinical trials, participants stressed the importance of establishing solid baseline measurements that document the natural history of HD. They also highlighted the importance of optimizing patient allocation and establishing realistic placebo controls.

And to monitor the trials as accurately as possible, participants ranked the identification of reliable biomarkers high on the list of priorities.

The plight of people like the HD-afflicted family, who generously shared their experiences with the workshop participants, provided a powerful motivation for identifying the most effective ways to search for a cure. With heart-wrenching clarity, the family illustrated how HD is a disease that devastates not only the individuals carrying the mutation, but entire families who must live with the specter of HD, generation after generation.

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