

# **Structural Studies of Huntingtin**

In memory of Max Perutz

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## **Abstract**

The complexity of the symptoms and pathology of Huntington's disease has long baffled researchers and hindered the development of therapies. Yet the primary cause of the disease is strikingly simple –an expanded stretch of glutamines in the huntingtin protein. Scrutinizing this fundamental source of pathology, participants at the “Structural Studies of Huntingtin” workshop discussed possible scenarios linking protein structure to disease, as well as options for rational drug design and drug screening assays. Most participants supported the hypothesis that polyglutamine tracts exist as random coils when huntingtin is in solution, regardless of their length. Much remains unknown about the structure of the full-length protein, and its formation of aggregates, however. Participants agreed that models, such as the water-filled nanotubes proposed by Max Perutz, require further testing. Some participants recommended obtaining full-length huntingtin, while others favored the study of minimal systems. Strategies to elucidate huntingtin's domains and protein partners were also discussed. In addition, participants considered potential mechanisms by which mutant huntingtin may cause cellular dysfunction, including the formation of ion channels, and huntingtin's potential involvement in nucleocytoplasmic transport. The development of multivalent compounds with high avidity for expanded polyglutamines was identified as a particularly promising opportunity for rational drug design.

## **A relentless disease**

Countless scholarly articles have described the progression of HD's symptoms in minute detail. Yet it is impossible to truly grasp the inexorability of the disease until witnessing it first-hand. A little over two years ago, participants at a Hereditary Disease Foundation (HDF) workshop were introduced to a young woman in her 20s suffering from HD's early symptoms. Except for a few occasional involuntary movements, she appeared healthy, describing the many sports she enjoyed, her multi-lingual abilities, her love of architecture, and her recent experiences as a model. She even performed several neurological tests designed to reveal subtle deficiencies with remarkable skill. Scientific knowledge aside, it seemed impossible that such a vibrant and youthful person would gradually succumb to the later stages of the disease. During this workshop, however, participants confronted the unforgivingness of HD. The same woman, now 29 years old, was racked by continuous movements, had difficulty speaking and swallowing, and required assistance for performing even the simplest of tasks, such as walking and eating. Her decline was heartbreaking. Yet her presence also nurtured hope. Thanks to her and her husband's generous participation, attendees were uniquely inspired to press on with the search for a cure.

## **The many faces of huntingtin structure**

### ***Poly-glutamine structure: Mounting evidence for a random coil***

The mechanisms by which poly-glutamine expansions in huntingtin lead to pathology remain uncertain, but several studies have proposed that a conformational transition occurs when the number of glutamines exceeds 36. Melanie Bennett and other participants, however, argued that, in solution, huntingtin's poly-glutamines most likely exist as random coils, regardless of their lengths. In support of this proposal, Bennett described her studies of huntingtin exon 1

using antibodies, as well as biochemical and biophysical techniques (Bennett *et al.*, in press). Bennett and her colleagues expressed and purified exon 1 constructs with 16 to 46 glutamine repeats. By including a C-terminus tag in their constructs, they were able to recover full-length proteins, under native conditions, and in milligram quantities. Also, fusion to thioredoxin helped keep the proteins in solution. Both circular dichroism and NMR spectroscopy yielded results that were consistent with random coil conformations for all constructs, regardless of their poly-glutamine lengths.

Bennett also relied on antibodies to examine poly-glutamine structure. Using analytical ultracentrifugation, her team assessed the stoichiometries of complexes formed between exon 1 and Fab fragments of MW1, an antibody previously shown to preferentially bind huntingtin bearing expanded poly-glutamine tracts in Western blots. They also performed biosensor binding studies, monitoring the interactions of exon 1 proteins with immobilized Fab fragments. Together, the results suggest that poly-glutamine behaves like a “linear lattice”, consistent with its existence as a random coil. In addition, the model indicates that multivalent ligands can bind expanded poly-glutamines with high avidity. As discussed later in this report, these findings have important therapeutic implications, potentially allowing the development of compounds with high specificity for pathogenic huntingtin.

Participants cited additional studies supporting Bennett’s findings. Tony Fink noted that studies from his lab show that many proteins, including  $\alpha$ -synuclein and histones, have unstructured domains, and these domains appear to be key for aggregation. Patrick Loll added that his studies of the interactions between ataxin-3 and the 1C2 monoclonal antibody also suggest there are no structural differences between normal and expanded poly-glutamines. Although performed under non-native conditions, the work of Ron Wetzel with peptides was also cited as supportive. In addition, Eric Altschuler noted that huntingtin’s poly-glutamine tract appears to function normally over a broad range of lengths –between 15 and 30. In contrast, the functions of highly structured domains, such as  $\alpha$ -helices, can often be dramatically altered by the insertion or deletion of a single amino acid. Bennett’s proposal also provides a basis for poly-glutamine expansion to result in a cytotoxic gain-of-function --by inducing aberrant interactions with other proteins, for example-- which is independent of protein aggregation.

Nevertheless, participants noted that the random coil proposal was not entirely proven. Bennett said that, although she had not found evidence for a  $\beta$ -hairpin or another known structural motif, her findings did not preclude the existence of a novel structure. Valerie Daggett also noted that what’s observed *in vitro*, may not accurately reflect what occurs *in vivo*. While studying a prion protein *in vitro*, for example, she observed a random coil which later proved to readily adopt a folded structure in the presence of copper. Fink added that the binding of protein partners *in vivo* could also induce conformational changes. Perhaps most importantly, all structural analyses to date have been limited to the study of huntingtin fragments. As noted by several participants, the structure of poly-glutamine tracts in the full-length protein may be more constrained. Indeed, Daggett noted that some studies of prion fragments proved of little value because they didn’t recreate the behavior of the full-length protein. In general, she cautioned, fragments can be much more prone to aggregation than their parent proteins.

Unfortunately, as pointed out by Carl Johnson, obtaining the 350 kDa, full-length huntingtin protein has proved difficult and will likely be an expensive and time-consuming endeavor. Investigators who have tried expressing huntingtin in bacteria and insect cells, such as Bennett, have run into problems with the formation of inclusions and low expression levels.

To bypass these difficulties, Fink asked if a minimum pathological fragment could be defined. As pointed out by Bennett, many researchers have relied on exon 1, which can reproduce many of the symptoms associated with HD in mouse models. Fink agreed that, based on his experience with protein aggregation and fibrillation, a soluble form of exon-1 is likely to report the key features of aggregation. Paul Patterson noted, however, that exon 1 was not chosen by rational design, and that poly-glutamine alone is much more toxic. An alternative would be to focus on recently identified huntingtin fragments with demonstrated physiological significance. Allan Tobin and Ethan Signer noted that several researchers --including Yvon Trottier, Vivian Hook, and Xiao-Jiang Li—have identified small fragments spanning less than 115 amino acids as key components of nuclear aggregates. Trottier's data suggest that the generation of one fragment, migrating at 55 kDa, might be a primary event in the formation of inclusions.

Yet another alternative is to bypass huntingtin altogether, and focus on a smaller, more manageable protein. In particular, Altschuler suggested using SCA6 or SCA3, proteins that, like huntingtin, cause neuropathology when bearing poly-glutamine expansions. Altschuler considered that this approach would be much more time- and cost-effective than working with full-length huntingtin. But Johnson and Nancy Wexler worried about the risk of these proteins yielding information that would not be directly applicable to HD. Signer suggested that solving the structures of two small proteins, and then searching for commonalities, might increase the likelihood of finding relevant data. Along these lines, Ray Truant proposed solving the structures of polyglutamine in SCA3 and mutant huntingtin exon 1 simultaneously.

Citing technology developed by his company, SGX, Michael Sauder proposed tackling huntingtin structure with a high throughput, industrial approach. Sauder explained that forward and reverse primers could be used to clone, in a proprietary fusion system, multiple pieces of huntingtin, in various expression systems, including *E. coli* and *Baculovirus*. The pieces could then be purified, crystallized, and modeled very rapidly using highly parallel techniques. For example, Sauder said that SGX had recently worked with an insoluble kinase which they processed from probe design to validated structure in only 28 days. SGX also has experience with complex proteins, such as the cystic fibrosis transmembrane conductance regulator (CFTR), which they are now working on.

Despite the known difficulties of dealing with full-length huntingtin, some participants recommended directing efforts towards its purification. Jeff Kelly suggested collaborating with Amgen, a company with ample experience in protein expression and purification. With the purified protein in hand, he said, nitrogen labelling and NMR could lead to the definitive elucidation of the poly-glutamine structure. Truant, on the other hand, proposed using traditional biochemical methods to extract huntingtin from natural sources, such as cow brains. Although several participants supported this idea, a potential limitation noted by David Eisenberg is that, *in vivo*, the protein might exist as a mixed population with varying levels of post-translational modifications, such as glycosylation, phosphorylation, and farnesylation. This heterogeneity could interfere with the use of mass spectrometry which, together with limited proteolysis, promises to be useful for examining huntingtin domains.

### ***Huntingtin aggregates: A multiplicity of structures?***

In addition to studying the structure of huntingtin as a single molecule, participants agreed that it is crucial to gain further understanding of huntingtin aggregates and how they form. As described by Fink, protein aggregation involves the initial formation of a partially-folded conformation with enhanced susceptibility to self-association (in many cases, the protein

regions mediating these initial interactions are relatively unfolded). The resulting soluble oligomers may be directly on the pathway to fibrils, or on a parallel path, leading to either larger soluble oligomers or amorphous deposits. The fibril path leads to the formation of stacks of molecules that form single strands, known as protofilaments. When two protofilaments intertwine they form protofibrils. At the next level of organization, 4 or 5 braided strands form a fibril.

If huntingtin aggregation proceeds through a simple nucleating reaction involving its N-terminus, Eisenberg noted that one might expect small fragments to induce nucleation more readily because of the increased number of ends per total protein. Kelly said that experiments with peptides seem to support this possibility, but aggregation may proceed quite differently *in vivo*. As noted by Fink, for example, the mechanism of aggregation may vary greatly depending on whether it occurs in bulk solution or on a surface, such as a membrane, which tends to accelerate the process. In addition, Kelly pointed out that, although nucleation mediated by single molecules has been described as a fundamental mechanism by which many proteins aggregate, there are other possibilities. Huntingtin may require, for example, a higher-order interaction, involving more than two huntingtin molecules, to form a seed. In addition, as described by Fink, other protein partners may play important roles in determining the progression of aggregation. His group has found, for example, that the natively unfolded protein  $\alpha$ -synuclein binds to histones (also disordered structurally) and forms specific complexes composed of both proteins that generate fibrils much more rapidly than  $\alpha$ -synuclein alone. This might be particularly relevant to HD, he noted, given studies suggesting the toxicity of nuclear aggregates.

Based on these considerations, Fink proposed the following hypothetical scenario. In the absence of particular protein partners, the N-terminal region of huntingtin is disordered. This lack of structure increases the probability of proteolysis and the consequent generation of fragments which, containing expanded polyglutamine repeats, have an enhanced propensity to aggregate. Aggregation may then be affected, positively or negatively, by additional factors, including interacting proteins. Genetic variability in these factors could affect disease progression, constituting a potential source for the observed phenotypic variations in patients carrying similar numbers of CAG repeats.

Participants also discussed other models of huntingtin aggregation. As described by Wexler, one of the early models of huntingtin aggregation, proposed by Max Perutz, suggested that poly-glutamine tracts aggregate into  $\beta$ -pleated sheets through the formation of polar zippers made by hydrogen bonding between amides. More recently, Perutz proposed that aggregation results in cylindrical  $\beta$  sheets that form water-filled nanotubes (Perutz *et al.*, 2002a). The model is particularly appealing because it suggests a structural basis for the 35-40 glutamine breakpoint that results in disease: two helical turns, each formed by 20 glutamines, constitute the minimum structure stable enough to form a nucleus for the growth of a helical fiber. A single turn can form with only 20 glutamines, but it is not stable.

Perutz acknowledged, however, that the model doesn't explain all his data, and other possibilities have been suggested. For example, Eisenberg described a model in which parallel strands, in register, form sheets which, in turn, fold over each other in an antiparallel manner. Participants agreed that since aggregates appear to be heterogenous, no single model is likely to represent the various forms that probably exist *in vivo*. This heterogeneity will make it difficult to test aggregation models. Kelly noted that measuring distances between protein components is very hard when working with a mixture of morphologies.

To understand the roots of the disease, Kelly stressed the importance of probing early intermolecular interactions. Studies of many diseases characterized by aggregation, he said, have focused on fibrils because they are visible. Yet patients with early symptoms of disease often have no fibrils. In the case of  $\alpha$ -synuclein, for example, it is likely that neurotoxicity arises from soluble oligomers which have the potential of rupturing membranes, said Fink. Furthermore, in HD, there is no clear correlation between the presence of visible inclusions and pathology.

Several participants expressed interest in seeing Bennett's studies extended to include aggregate analysis. Kelly suggested using heteronuclear NMR and electron spin resonance to test current models of aggregation. Daggett added that studies monitoring the concentration- and pH-dependence of aggregation were needed.

## **From structure to pathology**

### ***Protein partners***

Huntingtin's normal and abnormal functions are likely to be multi-faceted. As noted by Fink, large protein size is associated with structural requirements, multiple functions, interactions with multiple proteins, and functions that require repeated domains. Since huntingtin doesn't seem to be a structural protein or a multi-functional enzyme, Truant noted it is likely to be a scaffold for a variety of protein-protein interactions. Indeed, huntingtin has multiple HEAT repeats -- motifs found in several proteins that are often involved in mediating protein-protein interactions. In 1998, James Gusella and MacDonald described the presence of 10 HEAT repeats throughout the protein (Gusella and McDonald, 1998). And using a more sensitive algorithm, Sauder found even more: about 8 motifs between residues ~100-400 (instead of 3), and about 9 between ~750-1200 (instead of 4).

Several studies have searched for huntingtin's binding partners. In particular, the yeast two-hybrid system has revealed numerous proteins capable of interacting with huntingtin. Yet, surprisingly, all interactions identified to date map to the N-terminus of the protein. As discussed in the workshop, there may be additional, undiscovered interactions that have been missed because of the limitations of the yeast two-hybrid screen. On the one hand, noted Truant, the system tests interactions only within the micro-environment of the nucleus. New cytoplasmic versions of the two-hybrid system may yield additional candidates. In addition, as pointed out by Loll, two-yeast hybrid screens only identify binary interactions. Finally, because the HEAT repeats were not fully identified, the "baits" that have been used so far may have been prematurely truncated, said Sauder.

In addition to testing new baits, participants suggested several alternative techniques for identifying huntingtin partners. Signer wondered if fluorescence resonance energy transfer (FRET) might be used to probe cytoplasmic interactions *in vivo*. Truant proposed proteomic approaches, drawing attention to flag-tagging coupled with immunoprecipitation, and a cleaner technique, tandem affinity chromatography, or TAP-tagging, followed by mass spectrometry. Unfortunately, he noted that a company who pioneered the TAP-tag methodology, Affinium (Integrative Proteomics) in Toronto, showed little interest in his suggestion of pursuing huntingtin interactions. Although Truant said he provided them with information about the HD market, Wexler pointed out that many companies underestimate it, failing to realize that it is, in fact, larger than that of cystic fibrosis, for example.

A novel informatics approach, used by the company Protein Pathways, was described by Eisenberg. The method is based on the observation that proteins that interact with each other are

usually co-expressed. Thus, by surveying large numbers of microarray RNA profiling experiments to find patterns of co- and anti-expression, it is possible to identify candidate partners. According to Eisenberg, even entire pathways can be revealed using such analysis. Isaiah Arkin wondered, however, whether the technique would yield reliable results across different types of interactions, particularly, those involving unconventional proteins such as huntingtin. Bennett noted that the technique has proved useful not only for detecting stable interactions between housekeeping proteins, such as ribosomal subunits, but for identifying more transient associations as well. Indeed, Eisenberg noted that the approach has proven reliable enough to be used as a benchmark to assess the performance of the flag-tag and TAP-tag approaches described by Truant. According to this assessment, TAP-tag has an approximate success rate of 87%, whereas flag-tagging is only 8% accurate.

Participants agreed that, although using these techniques to find additional huntingtin partners will be important, it is also fruitful to analyze the interactions identified so far. Tobin, for example, noted that the study of vesicular proteins might be particularly revealing since the adult form of HD is characterized by aggregates in neural processes and there is evidence for huntingtin interacting with proteins of the secretory apparatus and the cytoskeleton.

### ***Huntingtin partners suggest role in nucleocytoplasmic shuttling***

Working with huntingtin yeast partners (HYPs), originally described by McDonald, Truant described evidence for huntingtin's potential involvement in nucleocytoplasmic transport. In particular, his group has focused on HYP-C, HYP-B, and HYP-L, proteins of the WW-domain class which had been previously linked to HD pathology. HYP-C contains two nuclear localization signals (NLSs), and is a nuclear speckle protein that also contains an arginine rich motif, or ARM, seen in several RNA-binding proteins. HYP-B, recently cloned by Brigitte Putzer's group, appears to be a transcription factor. HYP-L, also known as FIP2, is involved in binding kinesin motors via Rab8 signaling.

Truant set out to investigate these proteins after finding evidence for a functional nuclear export signal (NES) in huntingtin's C-terminus. The finding was puzzling because of the absence of nuclear import signals in huntingtin. Now Truant has found a possible explanation based on his discovery of a three-way interaction between HYP-C, HYP-L, and huntingtin that results in nuclear localization, and suggests huntingtin might have a normal role in the nucleus. Strengthening the evidence for a role of HYP-L in huntingtin transport, he observed that the rapid intracellular movements of a GFP-tagged construct of HYP-L closely match the dynamics of huntingtin micro-aggregates. Interestingly, his studies of SCA3 also indicate a potential link to the microtubule cytoskeleton.

Truant also presented data on SCA1 suggesting it is a nucleocytoplasmic shuttling protein. In particular, SCA1 appears to be involved in the specific export of mRNA encoding the glutamate GluR1 AMPA receptor. Building on this observation, Truant discovered that the clusters of poly-glutamine expanded SCA1 found in diseased cell nuclei, commonly described as 'inclusions', do not appear to be static deposits of misfolded proteins, but rather complexes that move, albeit more slowly than clusters of the wildtype protein. Together, these observations suggest SCA1 is a member of the family of neurologic RNA export defect diseases that include fragile X syndrome (FMRP), spinal muscular atrophy (SMN protein), and X-linked mental retardation (NTXF5). Truant also noted that ataxin-1 appears to be in the same sub-nuclear locale as HYP-C, which may imply that HYP-C has a similar function as ataxin-1. Truant plans to continue this work with emphasis on a comparative approach between SCA1 and huntingtin.

Intrigued by these findings, participants discussed the implications. Signer wondered whether knocking out huntingtin's NES would lead to disease. Truant said he is now making knockout and transgenic mice to answer this question, but he predicts the knockout will be lethal. Another issue brought forth by Altschuler was the implications of Truant's findings for HD being a gain-of-function or loss-of-function disease. Based on the SCA1 results, Truant speculated that poly-glutamine expansions may disrupt cell function by slowing down nucleocytoplasmic shuttling. Yet the observation that homozygotes and heterozygotes are similarly affected, said Altschuler, argues against a loss-of-function. Truant noted that another possibility is that toxicity arises from proteolytic cleavage, generating exon 1 fragments devoid of a NES which linger in the nucleus. Indeed, he noted that adding a NES to exon 1 greatly reduces toxicity and, conversely, adding a NLS increases toxicity.

### ***The channel hypothesis***

Yet another explanation for toxicity, presented by Bruce Kagan, is that poly-glutamine proteins form channels across the plasma membrane. Kagan first entertained the idea when he noticed that amyloid proteins are similar to microbial toxins, known as colicins, that form ion channels. Although at first he had trouble testing his hypothesis because of amyloid's tendency to aggregate, he has now observed channel formation in lipid bilayers using 7 different amyloid proteins.

The channels are large and very heterogenous. Kagan explained that, using hexa-fluoro-isopropanol to disaggregate the proteins before incubating them with the lipids, he observes more channel activities than he can count, making standard single-channel analysis impossible. The unit size of the channels, however, seems to depend somewhat on the proteins' degree of oligomerization –monomers generate smaller unit-sized channels that increase with time. Kagan has so far used a mixture of glutamine lengths, but even his studies with homogenous samples of other amyloid proteins have yielded very heterogenous results. He also noted that the channels are not highly selective, allowing movement of both divalent and monovalent cations. Kagan stressed he could not give a precise measurement of the size of the pores, but he estimates they are very large, about 10 Angstroms in diameter. In addition, they are not voltage-regulated and they appear to be continuously open. Except for zinc, all the channel blockers Kagan has tested have proved ineffective. Congo red seems to inhibit channel formation, but has no effect once the channels are formed. Poly-glutamine channels share these common characteristics, except that they are completely unaffected by either zinc or Congo red.

The findings are consistent with Perutz's general proposal of poly-glutamine proteins forming helical structures. Indeed, Kagan noted that a Japanese group has found that poly-glutamines above a certain length form channels, and have proposed this occurs through the formation of a  $\mu$  helix that spans the membrane, although no clear mechanism for the glutamine threshold for toxicity has been put forth. Also uncertain is the reason for the heterogeneity. Johnson noted that the key might lie in the length of the pore, rather than its width. Kagan agreed, adding that lengthening could increase flux by ensuring a full span of the membrane and avoiding lipids to pucker into the mouths of pores. Johnson further speculated that as proteins continue to oligomerize, beyond the length required to span the membrane, the channels' resistance would increase, reducing flux.

Arkin wondered how poly-glutamines might insert into the membrane since they are so polar. As noted by Eisenberg, one possibility is that the glutamines are completely hydrogen-bonded. Regardless of the mechanism, they appear to be very stably embedded in the membrane,

noted Kagan --once they're in, they don't come out even with extensive washing. Participants suggested performing additional tests to characterize this membrane interaction. Arkin suggested performing lipid flotation assays with radioactively labelled huntingtin. In addition, Kelly proposed measuring partition coefficients, although he noted that the heterogeneity may make this difficult. Arkin also noted it will be important to characterize huntingtin structure within the membrane. Kagan agreed, noting that, in addition to the hydrophobicity of the membrane environment, membrane thickness may impose unique structural constraints.

To further characterize channel behavior, participants suggested various approaches. Signer stressed the importance of extending Kagan's studies using a homogenous sample of uniformly-sized, monomeric poly-glutamines. To control initial conditions more tightly, Loll suggested using constructs with soluble fusion proteins that could be removed by proteases at time zero, allowing relatively synchronized insertion into the membrane. The process wouldn't be instantaneous, however, and as pointed out by Eisenberg, the fusion protein may not preclude membrane insertion. Altschuler suggested studying SCA6 because of its small size and because it has been shown to be a transmembrane protein. Participants also recommended moving beyond lipid bilayers. Arkin suggested the use of liposomes for greater flexibility, as well as oocytes to better approximate physiological conditions. As noted by Kagan, oocytes would have the added advantage of providing a built-in expression system.

Oocytes might also help address one of the greatest challenges presented by Kagan's findings: detectability. Several participants suspected that only a small fraction of total huntingtin may be recruited to the membrane, and within that fraction, an even smaller number of proteins might be directly responsible for causing toxicity. Since the baseline currents of oocytes are well characterized, Kagan expected these cells would be particularly useful for detecting small changes in whole-cell currents. Participants also suggested electrophysiological experiments in neuronal-like cells. Fink proposed using PC12 cells and Truant suggested using McDonald's clonal striatal cells derived from knock-in mice, which are easy to grow and transfect, and reproduce many of the hallmarks of HD pathology.

Determining whether channels actually form *in vivo* is also key. Altschuler asked if there were indications of heart problems or peripheral neuropathies associated with HD, or similar diseases, suggestive of abnormal channel activity. Although participants couldn't think of any, Kelly pointed out that, without a directed search, such clues are often missed. In addition, different cell types probably vary in their vulnerability to leakage, such that channel formation may have only minimal impact in most tissues. Kagan also pointed out that, although striatal cell function would probably be significantly disrupted by leakage, their sensitivity doesn't seem to be distinctive enough to explain their unique vulnerability to HD.

Truant suggested using immuno-electron microscopy (EM) to assess whether huntingtin can be found in membranes. In particular, he proposed using cryo-EM to increase the chances of antibodies recognizing their targets. Several clues as to where channels might be playing a pathological role could guide the search. As described by Kagan and Johnson, for example, Michael Levine's studies of cortical neurons in R6/2 mice show that these cells undergo transient, very large depolarizations. Tobin added that data from his lab in PC12 cells indicate that exon 1 affects neurotransmitter release which, he speculated, might be associated with channel formation. In addition, Kagan noted that, at least in the case of  $\beta$ -amyloid, channel formation may be affecting mitochondria, inducing the permeability transition that leads to apoptosis.

Kagan considered that the most effective tool for tracking and characterizing channels *in vivo* will be channel blockers. In addition, if channels are important in mediating pathology, blockers should have therapeutic potential. To identify such molecules, Kagan suggested using either lipid bilayers or liposomes to conduct a directed screen. Unfortunately, so far, there isn't much information to conduct a rational search. The channels' broad specificity does not provide many clues about the pores' charge or structure, other than suggesting it does not have a narrow neck like typical ion channels, explained Kagan. Tobin favored performing a broad, non-directed search with a large library of FDA-approved compounds, perhaps focusing on compounds that other HD researchers are already working on. But Johnson considered a more directed approach would be more fruitful. Signer wondered if trivalent cations, such as iron, might be worth investigating. He added that finding compounds that not only block, but are specific to, these channels would be particularly challenging.

## **Therapeutics**

### ***Rational drug design***

As noted by Tobin, the HDF's ultimate goal in probing huntingtin structure is to guide drug design and develop improved screens for therapeutic compounds. Participants agreed that poly-glutamine is an appealing drug target, particularly in light of Bennett's recent findings. As previously described, her data suggest that multivalent ligands could be used to target expanded poly-glutamines, while minimally affecting proteins bearing non-expanded repeats. The selectivity of the approach is key because, as pointed out by Truant, several recent articles demonstrate that huntingtin is required for neuronal health in the adult brain.

The ultimate effectiveness of such an approach will depend on additional considerations, however. Fink noted that small molecules will probably not be useful because the multiple binding sites will have to be significantly separated from each other. In addition, Patterson cautioned that the behavior of antibodies, on which Bennett's results are based, is complicated. The increased binding of antibodies, such as MW1, to expanded polyglutamines cannot be explained solely by valency. His studies indicate that even monovalent Fab fragments bind as much as 10-fold more tightly to mutated huntingtin than to the wildtype protein.

In addition, different antibodies, all recognizing polyglutamine tracts, yield very different staining patterns *in vivo*, suggesting that the full-length protein within cells has a complicated behavior that may differ in unexpected ways from exon 1's behavior *in vitro*. Patterson described three distinct staining patterns, including labeling of the Golgi, punctate cytoplasmic, and diffuse cytoplasmic, and noted that the patterns varied with cell type. Several possibilities to explain these results were discussed. Fink noted that the epitopes recognized by the antibodies may vary in length and that different protein partners, residing in different subcellular domains, may cover variable numbers of glutamines. Bennett added that the variability may stem from differences in the availability of glutamine side chains. Antibody cross-reactivity, said Loll, could also be contributing to the observed staining patterns.

Patterson noted that another possibility, with potential implications for therapeutic development, is the formation of induced structural variations. Not only might huntingtin's endogenous partners hold the protein in different conformations, which may be more or less recognizable by different antibodies, but the antibodies themselves may induce different conformations. If so, depending on their particular effects on structure, poly-glutamine binding compounds could either decrease or enhance toxicity. Indeed, although the underlying

mechanisms remain uncertain, Patterson said that two of his anti-poly-glutamine antibodies seem to increase aggregation and toxicity, whereas an anti-proline antibody reduces toxicity. Acknowledging these complexities, most participants agreed that it was nevertheless worthwhile to explore the potential of multivalent compounds that bind glutamine, and Bennett said she and her colleagues are currently working on their development.

More general considerations about structure-based drug design were also discussed. Stressing that expanded polyglutamine tracts lie at the heart of HD and other polyglutamine disorders, together with the importance of minimizing costs and time, Altschuler recommended focusing exclusively on polyglutamine. He noted that, at least for some diseases, such as spinal bulbar muscular atrophy, detailed knowledge of the mutated protein and its normal function had not been of much therapeutic help. In addition, proteins with very different functions have been shown to yield similar pathologies when carrying expanded polyglutamine stretches, suggesting the pathologic importance of these regions. Reiterating his suggestion for tackling structure with a smaller protein, Altschuler proposed focusing drug design on ataxin-3. Another suggestion for focusing on commonalities between polyglutamine diseases was put forth by Signer who proposed comparing protein partners across different polyglutamine proteins.

Participants also discussed potential ways to discourage huntingtin's adoption of pathological structures, and stressed the importance of attacking the early stages of huntingtin oligomerization. Kelly mentioned his work with other proteins indicating that small molecules can be used to prevent certain conformational changes, such as unfolding. But he added that such a strategy would be difficult to apply to huntingtin whose structure is undefined and may exist as an ensemble of different states. Signer asked about developing compounds to bind to transition states in order to shift equilibria, but Eisenberg said this was difficult to accomplish, even with better characterized proteins. Hoping to take advantage of previous studies, Tobin suggested examining compounds known to interfere with amyloid formation. Daggett and Fink mentioned several --including Congo red, anti-malarial compounds, doxorubicin, tetracyclines, and antibodies—and said they could put together a more comprehensive list. In addition, new anti-aggregating agents, particularly peptides, may emerge from Loll's ongoing screens in a SCA3 model system.

Johnson noted that the next wave of drug development must also be informed by huntingtin's function in the brain. An important issue that will need to be incorporated into the bigger picture is the finding that the pathology observed in HD's most visible target, the striatum, is probably due to the dysfunction of other brain areas rather than to the direct effects of mutated huntingtin on striatal cells. The concept derives from recent studies of chimeric mice showing that animals with a predominantly normal striatum seem to suffer from HD symptoms when other brain regions, including the cortex and thalamus, are mostly composed of neurons expressing mutant huntingtin. Conversely, animals with predominantly mutant striata remain relatively healthy, if other brain areas are normal.

A few proposals departed from the workshop's general focus on huntingtin protein. Truant, for example, suggested targeting the CAG repeat expansion in huntingtin mRNA. He noted that RNA as a target is appealing for several reasons: it adopts a three-dimensional structure based on sequence that can be easily determined by NMR or crystallography; the target resides in the cytoplasm, which is less of a pharmacological challenge; the half-life of RNA is much less than that of protein, allowing higher effective turnover of a drug in the cell; and finally, an anti-CAG drug that inhibits translation could be effective against all CAG repeat

diseases. Truant has submitted a proposal to the HDF describing a high throughput screen based on this approach.

Truant also suggested exploring treatments to regulate body mass. During the patient presentation, Wexler noted that heavier individuals seem to suffer from uncontrolled movements less than thinner patients. Based on these comments and recent studies showing adipose tissue's production of neuroleptic hormones involved in body mass homeostasis, such as leptin, Truant wondered if purified extracts from adipose tissue might ameliorate disease progression. He proposed using mouse models to monitor the extracts' effects on HD symptoms, including the wasting phenotype observed in some models. Interestingly, a recent study by Michael Hayden showed that the only defined phenotype of mice deficient in huntingtin-associated protein 1, HAP1, is a lack of appetite.

### ***Drug screening***

To find drugs to combat HD as efficiently as possible, the HDF is currently funding a broad range of drug screening assays designed to enable hierarchical testing. Molecular tests that monitor nucleation, oligomerization, and the formation of large, macroscopic aggregates, explained Johnson, are being used to identify lead compounds that inhibit aggregate formation. In addition, a wide variety of model systems --including yeast, *C. elegans*, *Drosophila*, PC12 cells, and striatal cells that have been immortalized with the large T antigen— are providing toxicity, cellular dysfunction, as well as aggregation read-outs. In these *in vivo* systems, throughputs range from 100,000 drugs per week, in the case of yeast and *C. elegans*, to 10 drugs a week, in the case of *Drosophila*. At the narrow end of the funnel, the HDF is funding five labs to develop screens in mice. These are expected to assess 100-150 drugs per year.

To illustrate these efforts, Johnson described two assays developed by Aurora/Vertex San Diego. The first relies on labeling the MW4 antibody, which preferentially binds to huntingtin harboring long stretches of glutamines, with a fluorescent tag to monitor its interactions with GFP-tagged 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. Compounds that bind to the poly-glutamine stretch of exon 1 will block the binding of the antibody, and thus prevent FRET.

The second assay is designed to monitor the cleavage of huntingtin and its ability to get into the nucleus. 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, is used in this assay. 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. Compounds that block this activation are expected to yield protease inhibitors, as well as inhibitors of exon 1 nuclear transport.

In both assays, compounds whose effects are three standard deviations above the mean of all compounds being tested are selected as initial hits. So far, the antibody assay has yielded approximately 800 compounds that have met this criteria and produced reproducible results. Johnson expects the number will be narrowed down to 400 after obtaining dose-response curves. Secondary screens to assess prevention of toxicity will subsequently be performed in PC12 cells. Because mouse model throughput is very low, candidates will have to be whittled down extensively before reaching this stage.

Participants generally agreed with this hierarchical approach. Concerned about straying too far from physiological relevance, however, Patterson stressed the importance of using toxicity as a key readout. In addition, Altschuler advocated conducting as much of the screening process on mouse

models as possible. Signer noted that chemical manipulations can increase the potency of promising candidates considerably, so it makes sense to screen and process drug leads at least as far as this stage before using animal models. He also suggested that mouse throughput might be increased by pooling compounds, administering 4 or 5 drugs simultaneously. Another possibility is to raise the bar for identifying initial hits. Tobin cautioned, however, that because it is not yet clear which of the many screening assays will prove effective, it is probably best to test several assays before limiting candidate subsets too much.

A particularly promising approach described by Johnson is a massively parallel screening effort --led by the NINDS and involving multiple groups, including the HDF-- testing hundreds of FDA-approved compounds, using 29 different assays, for their effects on various neurodegenerative diseases. Fourteen of the assays are designed to identify leads for polyglutamine diseases. As Tobin explained, this broad, blind screening effort has unprecedented power, similar to that of an unbiased, large-scale genetic screen. Based on the group's first meeting a month ago --after 6 months of work, screening roughly 1000 compounds-- Johnson summarized the project's status. The ideal scenario of finding a few compounds that work well across many assays, at low concentrations, has not materialized. Some researchers even thought there were no statistically significant hits across assays. However, the data are still being analyzed and some encouraging hints have already emerged. Johnson noted that three compounds with the same mechanism of action had yielded positive results at low concentrations (~50 nM). In addition, results from a few other compounds, with non-overlapping mechanisms of action, were also encouraging, said Tobin, suggesting the existence of various potential avenues for tackling the disease. The next step will be to focus on a subset of standardized assays to reduce variability in assay quality.

Several suggestions to improve and extend current screening efforts were also discussed. Despite the moderately encouraging results with FDA-approved drugs, Fink considered that the chance of this pool yielding very effective drugs was small, so he suggested tapping into companies' private libraries, including combinatorial ones. He also noted that tests on non-human primates may need to be included in the drug screening process. Signer said that two groups, one using adenovirus and the other using a lentivirus, had explored the possibility of creating primate models of HD, but he was uncertain about the current status of their projects. Altschuler expressed concerns about spending too much money and effort on drugs that might be unable to cross the blood brain barrier. As noted by Minka vanBeuzekom, however, many powerful new methods for drug delivery are being developed. For example, Patterson described the use of 'intrabodies,' antibody domains that can be expressed within cells, secreted, and engineered to carry a sequence that allows them to move across membranes.

### **A few final thoughts**

There is clearly no lack of ideas to accelerate HD research and the search for a cure. A key challenge lies in reconciling the multiple theories of huntingtin's normal function and toxicity, and prioritizing drug design and screening efforts. Truant noted that, from his perspective, disease relevance should be more strongly emphasized since many studies tend to digress away from the human disease and, in some cases, even from the biology of huntingtin and HD. Another major challenge lies in translating suggestions, comments, and recommendations into experimental results. Wexler urged participants to suggest ways for the HDF to follow up on participants' proposals. Eisenberg added that by acting as a centralized

source of reagents, such as synthetic poly-glutamine chains and exon 1 constructs, the HDF could facilitate research.

Participants also discussed the need for establishing guidelines for the handling and disposal of materials in labs working with mutated huntingtin and other aggregating proteins. As pointed out by Bennett, based on what's known about prions, these proteins may also be transmissible, at least to some extent. Indeed, Daggett drew attention to an article showing transmission of systemic amyloidosis by a prion-like mechanism (Lundmark *et al*, 2002). To help labs deal with this potential hazard, vanBeuzekom said she would look into drawing up a set of recommendations.

Finally, Wexler urged participants to take action against, what could be, a truly restrictive barrier for the development of therapies to treat neurodegenerative diseases. Wexler distributed a letter written by Fred Gage on behalf of the Society for Neuroscience prompting neuroscientists to oppose legislation that would ban therapeutic cloning. In summary, Gage asks for support of S. 2439, the Human Cloning Prohibition Act of 2002 introduced by Senator Specter, which permits therapeutic cloning with safeguards, as opposed to the legislation introduced by Senator Brownback, which prohibits all forms of cloning. Wexler enjoined participants to contact their government representatives and encourage their institutions to get involved.

#### **List of Action Items:**

1. Structural analyses
  - a. Set up collaboration with Amgen to express and purify full-length huntingtin (Kelly)
  - b. Apply GSX industrial approach to solve structure of full-length/fragments of huntingtin (Sauder)
  - c. Solve structure of small polyglutamine protein, such as SCA3 or SCA6 (Altschuler)
  - d. Isolate full-length huntingtin, using traditional biochemical techniques, from a natural source (Truant)
  - e. Examine huntingtin structure in membranes (Arkin)
2. Aggregate analyses
  - a. Use heteronuclear NMR and electron spin resonance to test current models of aggregation (Kelly)
  - b. Characterize the concentration- and pH-dependence of aggregation of exon 1 (Daggett)
3. Protein partners
  - a. Mine RNA profiling data (Eisenberg; Protein Pathways)
  - b. Apply TAP-tagging followed by mass spectrometry (Truant; Affinium)
  - c. Design yeast two-hybrid baits avoiding truncation of HEAT domains (Sauder)
  - d. Use flag-tagging coupled with immunoprecipitation (Truant)
4. Nucleocytoplasmic shuttling
  - a. Continue Truant's studies probing huntingtin movement and compare with SCA1 (Truant)

- b. Assess effects of knocking out huntingtin's NES (Truant, Signer)
5. The channel hypothesis
- a. Identify channel blockers (Kagan) by conducting large-scale screens (Tobin) and/or pursuing rational design (Johnson). Test trivalent cations (Signer).
  - b. Extend Levine's electrophysiological studies of cortical neurons in R6/2 mice (Kagan)
  - c. Conduct lipid flotation assays (Arkin) and measure huntingtin partition coefficients (Kelly)
  - d. Extend Kagan's bilayer studies using uniformly-sized, monomeric polyglutamines (Signer, Kagan)
  - e. Extend Kagan's studies to include huntingtin fragments, constructs fused to a protease-removable, soluble protein (Loll), and SCA6 (Altschuler)
  - f. Extend Kagan's studies to other model systems, including liposomes (Arkin), oocytes (Arkin), PC12 cells (Fink, Tobin), and McDonald's clonal striatal cells (Truant)
  - g. Search for evidence of huntingtin's association with membranes using cryo-immuno EM (Truant)
6. Rational drug design
- a. Develop multivalent ligands that preferentially bind to expanded polyglutamines (Bennett)
  - b. Target CAG repeats in RNA (Truant)
  - c. Analyze the epitope-recognition sites of Patterson's antibodies (Patterson)
  - d. Focus efforts on small polyglutamine protein, such as ataxin-3 (Altschuler)
  - e. Compare protein partners across polyglutamine proteins (Signer)
  - f. Examine compounds previously shown to interfere with amyloid formation (Tobin; Daggett; Fink)
  - g. Test compounds that emerge from assays searching for SCA3 anti-aggregating agents (Loll)
  - h. Assess potential benefits of adipose tissue extracts (Truant)
7. Drug screening
- a. Pool drugs to enhance mouse model throughput (Signer)
  - b. Adjust initial hit threshold to balance the need for limiting the number of compounds to be tested in mice with the risk of losing potentially efficacious compounds (Signer, Tobin)
  - c. Reduce variability in assay quality in NINDS-led screening project (Johnson)
  - d. Extend drug screens with private company libraries (Fink)
  - e. Examine potential use of non-human primate models (Fink, Signer)
8. Miscellaneous
- a. Establish safety guidelines for handling of aggregating proteins (vanBeuzekom)
  - b. Support efforts to avoid the banning of therapeutic cloning (all participants, Wexler)
  - c. Establish centralized source of key reagents (HDF, Eisenberg)

- d. Submit suggestions to HDF for improving follow-up of ideas generated at workshop (all participants, Wexler)

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