Workshop Report: Huntingtin protein and the cytoskeleton
Nov 18 & 19, 2010
CHDI, Inc., New York
Prepared by Stephani Sutherland, PhD

Huntington's disease (HD) arises from a mutation in the gene for a protein called Huntingtin (Htt). The mutation takes the form of an expanded polyglutamine region in the protein, which may have important effects on the protein's structure and therefore its function. The initial focus in HD research was to understand the toxic gain of function introduced by the mutant protein, which forms aggregates that accumulate in neurons. More recently, research has shifted focus to understand the basic natural functions of the wildtype (WT) Htt protein as well. While mutant Htt protein does indeed introduce toxic effects, it appears more and more likely that interference with or loss of the functional WT protein also contributes to neuronal dysfunction. On November 18 and 19, 2010, CHDI hosted a workshop in New York to assess our understanding of the role of both WT and mutant Htt in cellular functions specifically linked to the cytoskeleton. The workshop originated from two basic, complementary ideas that provided the basis for much of the workshop's discussion. First, various lines of evidence from workshop participants and others suggest that the Huntingtin (Htt) protein forms an integral component of protein complexes that coordinate cell trafficking and may represent a control point for regulation. Second, recent work from Nancy Kleckner describes the mechanical properties of protein HEAT repeat regions. Huntingtin contains these domains, which might confer important mechanical properties that are likely integral to the protein's role. The following is a list of broad ideas discussed during the course of the workshop.

1. Huntingtin (Htt) contains between 36 and 80 HEAT repeat regions, which may confer complex mechanical properties and have major implications for the protein's role in cells. HEAT-repeat regions could allow Htt to act as a mechanically dynamic scaffold for other proteins. HEAT repeat regions allow for re-distribution of stress throughout large molecules and provide a built-in communication system between distant parts of the protein and its interacting partners.
2. Htt contains many sites for post-translational modifications, including highly conserved phosphorylation sites with important effects on protein function. For example, phosphorylation of Htt switches the protein's affinity preference from actin to tubulin in both wildtype and mutant full-length Htt. These sites are under-phosphorylated in the disease protein, and phosphorylation appears to be protective.
3. The various proteins that associate with Htt are sure to be key to their multiple potential functions in trafficking. Htt forms distinct, direct and indirect interactions with the dynein/dynactin and kinesin motor complexes; these interactions are influenced by Htt's phosphorylation state.

4. Vesicles move in the anterograde direction using one to two molecules of kinesin and in the retrograde direction using six molecules of dynein, with what amounts to roughly equal and opposing forces. Regulation of these motor proteins is likely to determine which motor protein wins the "tug of war" and may affect motor movement speed and direction. It's possible that Htt acts as a dynamic scaffold to coordinate interactions between vesicles, filaments, and these motor proteins.
5. Loss of WT Htt or expression of mutant Htt results in reduction of vesicular trafficking by about 30%. While this reduction may not be immediately detrimental to cells, the resulting loss of trophic support and signaling could underlie cells' eventual decline.
6. Energy demands and other conditions specific to certain cells (and particularly medium spiny neurons) may render them more vulnerable to the non-specific defects brought on by mutant Htt. Transport defects particularly of mitochondria and neurotransmitter receptors may contribute to cell dysfunction and death.
7. KineMed, Inc., has developed techniques to measure the kinetics of arguably any protein that can be isolated from bodily fluid from synthesis to secretion. Using a standard proteomics approach augmented by labeling with heavy water and specialized mass spec analysis, they can see a dynamic picture of protein processing rather than a still snapshot. Using this approach, they found that neuronal transport of cargo proteins is impaired in several models of motor neuron disease. In addition, the plasticity or "dynamicity" of microtubules in the models increased dramatically compared to controls. The dynamic proteomic technique may be useful in both finding an HD biomarker and in pursuing therapeutic avenues.
8. The actin cytoskeleton is emerging as an active and dynamic component of cellular processes like endocytosis and exocytosis rather than a static support structure.
9. Cofilin acts to dynamize actin filaments, providing plasticity in the cytoskeleton. Cofilin can also stabilize actin into rods, which may be a marker of neurodegenerative diseases. Htt binds cofilin and is found in actin-cofilin rods in the nucleus. Paradoxically, rod formation can also be neuroprotective in that it sequesters cofilin and slows down actin turnover, which consumes ATP.
10. In general, protein-protein interaction studies need to be conducted using the full-length Htt protein to glean information about the protein's biology. Saudou's new pARIS construct may aid in these efforts, providing a synthetic cDNA of the full-length human Huntingtin (Htt) sequence. The effort to develop the pARIS construct arose because of the difficulty to make specific modifications to the full-length protein and, as discussed at length, because of problems with using protein fragments.

Overview

The workshop began with an overview of what we know about the Huntingtin (Htt) protein and progressed to a discussion of the nature of HEAT repeat regions, how these sequences might confer mechanical properties, and how this might affect Htt's function in cells. Their combined experience allowed participants to form a complex but incomplete picture of what roles the Htt protein might play in cellular functions related to the cytoskeleton. In addition to discussions of data, the workshop also tended towards the underlying (sometimes philosophical) issues about how to approach neurodegenerative disease questions in general. A major current running through the workshop was the relatively recent shift in the HD field from viewing HD pathology as resulting exclusively from toxic mutant Huntingtin—and perhaps buildup of aggregates—to the more current focus on trying to understand the natural function of the wildtype protein and how that might be disrupted in disease as well. Workshop participants also took on the question of selective vulnerability. Ethan Signer made the important point that HD is a disease not of cell death but of cell dysfunction, which is very different. Early in disease, synaptic function is impaired. With loss of functional synapses comes withdrawal of processes and, perhaps, eventually cell death. But to approach the problem from the perspective of maintaining cell function rather than purely survival requires a different strategy. Participants agreed that Htt likely has many diverse functions and may act as a large, complex machine. But they disagreed as to whether "every complex machine has a single on-off switch," as Ray Truant asserted. Nancy Kleckner and others argued that even if you define all of Htt's functions, "it's not going to be *one thing*" that can be shut off to avoid HD pathology. Unless, of course, the on-off switch can shut down production of mutant Htt. This outcome would likely be reached only through a genetic approach, which comes with its own set of complications. Together, these factors suggest a disease mechanism that makes sense: while mutant Htt confers a toxic gain of function, it appears increasingly likely that wildtype Htt plays a vital role in cellular processes whose disruption exacerbates disease. The importance of this role might vary with cell type, and loss of wildtype (WT) Htt may not have overt consequences for years—which would explain the late onset of disease. Allan Tobin declared that he likes this explanation for late onset better than the "old" paradigm, which held that neurons don't lose function until slowly accumulating aggregates form and disrupt the cell. The tools and lines of research developed by the participants are sure to shed more light on this fascinating protein in the coming months.

What do we know about Htt's structure, and how might HEAT repeat regions contribute to potential mechanical properties of Htt?

Huntingtin (Htt) is an extremely large protein at over 350 kD with over 3000 amino acids. The mutation in Htt that leads to Huntington's disease (HD) arises from a poly-glutamine (poly-Q) expansion encoded by a series of CAG repeats. In the

wildtype (WT) protein, this region contains 35 or fewer glutamine (Q) residues, with a mode in the human population of about 23. Expansion to 39 or more Q residues results in HD, while disease is variable between 36 and 38 repeats. Disease onset, defined neurologically by motor signs, accelerates with increasing length of this poly-Q region. Structural analysis of WT Htt has revealed that the protein may exist in many different conformations. The protein's main structural elements can be classified into regions that are highly conserved (between all species) and form either alpha helices or beta strands, or as more structurally variable, non-conserved regions. In general, these less-conserved "random coil" areas contain post-translational modification sites for phosphorylation, palmitoylation, and nuclear localization among others. Randy Singh, a postdoc in Marcy MacDonald's lab at Harvard, shared their structural analysis based on these classifications. Next, the lab will focus on structural elements of Htt proteins that vary according to Q length.
Htt contains many HEAT repeat regions, not all of which have yet been identified; estimates range from 36 to 80. By nature, HEAT repeat regions are hard to detect with algorithms and instead have emerged (in other proteins as well) from careful analysis of the protein structure rather than sequence. HEAT repeats work in pairs not only to bring molecules into close proximity to one another, i.e. as a static scaffold, but also to allow them to move relative to one another in complex ways, thereby forming a mechanically dynamic scaffold. Higher-order properties of these scaffolds may emerge with increasing HEAT repeats throughout a molecule.
Nancy Kleckner of Harvard University shared her understanding of HEAT repeats based on extensive work that she's done with the PR65/PP2A protein complex, especially in the framework of chromosome and spindle dynamics in dividing cells. HEAT repeats allow stress to be redistributed along the entire structure of a molecule. As an analogy, imagine a rubber band pulled tight: it's under tensile stress. When you let go, the band doesn't simply relax in one place; the stress is redistributed with effects throughout the rubber structure. For Htt, this implies that HEAT repeat regions located throughout the molecule may have mechanical implications for the entire protein. Kleckner explains, "Everything is possible once you think of (Htt) as a mechanical molecule." It gives the entire system a built-in method for communication, whereby events at one part of the molecule have effects on the entire protein and on all its associated molecules. Thermal forces are likely sufficient to activate the mechanical properties contained in HEAT repeats. You can imagine "gazillions" of scenarios, Kleckner says, that may be contained within a mechanical protein. Between this new understanding of the potential for mechanical properties and what we know about the Huntingtin structure, a picture of the protein emerged. This giant molecule likely has a flexible spring-like structure—but it's "stiff," not "floppy." Such a protein could provide a scaffold platform that's "squishy" and can act as an elastic shock absorber rather than "a bowling ball." So are these HEAT repeats in Htt the site of its interactions with other proteins? The evidence to date suggests that protein interactions may occur at, or at least be influenced by, the regions in between HEAT repeats.

What proteins does Htt bind to in terms of cytoskeletal elements, including motors, filaments, and other trafficking components?

The various proteins that associate with Htt are sure to be key to their multiple potential functions in trafficking. What are some of these proteins and what do we know about them? Htt forms a direct interaction with dynein, and forms indirect interactions with dynactin and with kinesin through Huntingtin-associated protein 1 (HAP1). Htt appears to form a stress-dependent interaction with cofilin, an actin-dynamizing protein. Profilin, optineurin, and various myosins are also included among the proteins that interact with Htt.

The literature also points to Htt binding directly to actin and tubulin, but it's unclear how specific those interactions are with such abundant cellular proteins. Of these molecules, dynein appears to be perhaps most critical to transport function. Dynein drives the transport of brain-derived neurotrophic factor (BDNF), is required for autophagy, and affects exocytosis and endocytosis.

Erika Holzbaur at the University of Pennsylvania studies the forces required to move vesicles along their filamentous tracks in neurons. Stall force describes the amount of force required to keep a motor protein from walking along a filament and can be measured with *in vitro* assays. Late endosomal vesicles appear to move in the anterograde direction using one to two molecules of kinesin (which has a stall force around six picoNewtons, pN) and in the retrograde direction using six molecules of dynein (with a stall force of only about one pN per molecule). Regulation of these motor proteins is likely to determine which motor protein wins the "tug of war" and may affect motor movement speed and direction.

Proteins that link Htt to vesicles may be key as well. Arun Pal, a postdoc with Marino Zerial at the Max Planck Institute in Dresden, looked for proteins that interact with Rab5, an important regulator of early endosome vesicular transport, and turned up an interaction with Huntingtin-associated protein 40 (HAP40). HAP40 binds at the C-terminus of Htt, bridging it with Rab5-positive vesicles. With either mutant Htt or overexpression of HAP40, Rab5 vesicles no longer move processively and move more slowly. In addition, expression of TrkA and TrkB receptors for neurotrophic factors declines severely. Mice with knocked-out HAP40 survive and breed, but have not yet been fully characterized. Pal hypothesizes that when endosomes don't move properly, it affects signaling transmission. In related work, Truant has seen stress-dependent increase in nuclear HAP40; it's not yet clear if this is connected to Htt or not. Both Htt and HAP40 can independently enter the nucleus.

Holzbaur stated the need for more specific information on the biological sites of these interactions. She suggested a move away from big protein deletions to try to predict the sites more precisely. (Fred Saudou's pARIS-htt construct should be useful to help understand small regional interaction sites and their effects in cells.) Alex Kiselyov of CHDI, with an eye to drug design, says when it comes to therapeutic strategies, "all confirmed protein interactors and their sites of action should be taken seriously in terms of structure."

How might these structural considerations and post-translational modifications alter Htt's function in terms of trafficking?

Htt contains many sites for post-translational modifications, including phosphorylation sites that are highly conserved (within mammalian species, at least). These sites have important effects on protein function. Kleckner noted that clusters of phosphorylation sites could produce "big balls of charge" that may themselves produce mechanical effects. Ray Truant of McMaster University in Ontario, Canada, has been focused on understanding the effects of these modifications, particularly in the protein's first 17 amino acids. Using FLIM-FRET imaging, Truant saw Htt switch its affinity from actin to tubulin. The technology, more properly called fluorescence lifetime imaging measure (FLIM) of Forster resonant energy transfer (FRET), uses fluorescent molecules in a non-concentration-dependent method to see protein-protein interactions inside live cells at a level of resolution down to 8 nm. Truant saw that phosphorylation at two serine sites in this 17-amino acid region switches the protein's affinity preference from actin to tubulin in both wildtype and mutant full-length Htt. Truant explained, "phosphorylation definitely affects this affinity in a binary manner: on, off." These sites are under-phosphorylated in the diseased protein, and phosphorylation appears to be protective.

What are the roles of wildtype (WT) Htt in motor-driven neuronal transport, and how does the mutation affect these functions?

Fred Saudou of the Institute Curie in Paris has devoted much of his research career to answering these questions. Work from his lab and others have made clear by now that Htt plays an important role in vesicular trafficking *in vivo*. However, although many ideas and assays being discussed focus on transport, this may only be a marker for Htt's true role in the cell, which could involve endocytosis or protein secretory processes among other possibilities. In neurons expressing mutant Htt, vesicular traffic is reduced in both directions, both in velocity of transport and with fewer vesicles on the microtubules (MT). In mouse, loss of Htt function reduces vesicular transport velocity by about 30%. This same reduction arises from either expression of mutant Htt or from knockdown (by about 90%) of wildtype Htt. The result suggests that Htt is necessary for normal transport levels is but not required for all transport per se, and that mutant Htt has a dominant effect. Transport is similarly reduced in neural stem cells from HD patients with a 50 Q repeat, where reduction of expression of mutant Htt restored transport function. Perhaps the most important functional consequence of this reduced transport is the loss of trophic support from BDNF.

When WT Htt is reduced using siRNA, cells lose the compaction of the Golgi network and transport efficiency is reduced; if Htt is re-expressed, cells re-form the Golgi and trafficking is restored to normal levels. So Saudou conducted the following proof-of-concept experiment using his pARIS construct of full-length human Htt protein. In Htt-silenced cells, they re-expressed versions of Htt that are unable to bind dynein or HAP1 (which binds dynactin). In either case, these cells were not able to reform

the Golgi or to restore vesicular transport. So it appears that strong functional interactions between Htt and dynein and between Htt and HAP1 are required for normal vesicular transport, and these interactions are disrupted by the Htt mutant. Furthermore, increased phosphorylation at Serine 421 of mutant Htt protein appears to reform the natural protein interactions, because it restores trafficking and transport of BDNF in neurons.

How does Htt regulate trafficking of organelles along the cytoskeleton, and how might mutant Htt alter energy supplies at dendritic spines?
Some participants wondered, even with defects in BDNF transport, if 70% of traffic remains intact, is the defect enough to cause the massive damage seen in HD? Saudou argues that it is, and that once again this might point to why HD is such a late-onset disease. The transport defect is evident right away, but this reduced level may be sufficient for cells to function under quiescent conditions. Once stressors accumulate, however, the reduced availability of trophic factors may not be enough. Likewise, TrkB receptors may be reduced in the membrane, leaving the cell unable to use the available BDNF. And you can imagine other cumulative effects of impaired transport over time. Jim Zheng pointed out that glial cells might represent an alternative source of BDNF in the striatum.

What is the role of Htt in terms of accumulation of actin-cofilin rods?
The actin cytoskeleton is emerging as not only a structural support mesh for cells but also an active participant in aiding vesicle endocytosis and exocytosis. Jim Bamburg of Colorado State University presented a crash course in cofilin-actin dynamics. Cofilin is the primary protein responsible for dynamic remodeling of actin; that is, it enhances the turnover of actin subunits. Cofilin's effect depends on local conditions. When globular (g) actin subunits are available for incorporation, filamentous (f) actin polymerizes and grows. If actin-sequestering proteins are present, however, cofilin promotes depolymerization. Cofilin binds actin in its twisted confirmation, which can lead to stabilization of actin filaments and eventually to severing of filaments into small, stable pieces. Self-association of cofilin-saturated actin filaments forms bundles, also called rods. Rods block axonal transport, cause loss of synapses, and cause atrophy of neurites distal to where rods form, leading to loss of synapses. Under certain stress conditions, rods may form in the nucleus, where they have been shown to co-localize with the Htt protein. Accumulation of rods can be reversible and appears to be an early step in Alzheimer's disease (AD) pathology. Multiple types of stress evoke rod formation, particularly depletion of ATP, because cofilin preferentially binds the ADP-bound form of actin. As a cell's ATP:ADP ratio falls, more ADP-actin is produced—the form that gets incorporated into disruptive rods. Cofilin is enriched in areas of rapid cytoskeletal growth and turnover, such as growth cones. Both kinases and phosphatases are important regulators of cofilin, and likely achieve their opposite effects by distinct sub-cellular localization.

So how do rods fit with our understanding of the Htt protein? The mutation's acute effects on cell transport under resting conditions may not tell the whole story. Stress conditions (which accumulate with age) may bring on slower, long-term deficits. Truant found the Htt-rod connection in his investigation of Htt's first 17 amino acids, which is known to be involved in ER tethering. Cellular stress caused a release of Htt from the ER and localization to the nucleus, where he found it was incorporated into actin-cofilin rods. Although cytoplasmic rods also form in response to stress, Htt is contained only within nuclear rods. In cells expressing wildtype Htt, nuclear rods dissipate rapidly after stress relief. But with mutant Htt, the rods persisted in the nucleus long after conditions were returned to normal. When endogenous Htt protein was knocked down, cells were more sensitive to stress and formed these so-called persistent rods.

What might be the effects of mutant Htt on cytoskeletal dynamics, and how could disrupted dynamicity influence neurodegenerative disease processes?
In cells from HD patients at various stages, Truant saw that cofilin was increasingly trapped into a cross-linked state with actin. Formation of Htt-containing persistent rods requires a cellular stress and cross-linking activity by trans-glutaminase 2 (TG2). This provides a mechanism for the observation that TG2 activity is elevated in HD. Because rods are a feature of so many neurodegenerative diseases, it raises the question of how they differ in each situation. Rather than a cause of pathology, rods may be an early indicator of misregulated actin dynamics or a marker of stress in general. The specific effects of nuclear accumulation of rods (which contain Htt) remain to be seen.
And, Bamburg explained, not all effects of rod formation are harmful. Free cofilin likely forms disulfide bonds under stress conditions that prevent its binding to actin and promote binding instead to the mitochondrial membrane, thereby activating release of cytochrome C and promoting apoptotic pathways. So while rod accumulation is synaptotoxic, rods can sequester this potentially harmful free cofilin, thereby providing neuroprotection. In addition, cofilin promotes actin turnover, which is costly in ATP. With less cofilin dynamically turning over actin, the cell's requirement for ATP is reduced, allowing it to recover from stress.
Marc Hellerstein and Patrizia Fanara of KineMed, Inc., in California explained their techniques to measure the kinetics—from synthesis to secretion—of arguably any protein that can be isolated from bodily fluid. Using a standard proteomics approach augmented by labeling with heavy deuterated water and specialized mass spec analysis, they can see a dynamic picture of protein synthesis, transport, and breakdown rather than a still snapshot. Hellerstein likened the approach to using a video camera rather than a still shot, for example of freeway traffic. A still shot doesn't carry nearly as much information, for example about how fast the cars are moving. Using this approach, they found that the plasticity or "dynamicity" of microtubules in several models of motor neuron diseases was increased dramatically, from about 1% turnover or less in healthy mature neurons to as much as 40% turnover of microtubules in 24 hours. So while Htt's associated proteins

should be considered as viable targets, perhaps so are the microtubules themselves. In addition, the transport of protein cargo can be monitored in the living brain. Neuronal transport has been shown to be impaired in neurodegenerative diseases in both humans and experimental animal models. More immediately importantly is the potential for the technique to uncover a useful biomarker of HD—a biological molecule that reliably changes with disease progression. Perhaps changes in neuronal transport of selected protein cargo molecules will be that marker.

How might Htt alter availability of postsynaptic receptors (like AMPA) and mitochondria?

Jim Zheng of Emory University points out that the dendritic spine is the most complex part of the neuron, at least in its structural plasticity and the energy requirements that come with it. To cope with this energy demand, mitochondria reside right at the branch points of spines, supplying ATP. In Zheng's studies of AD, neurons become more vulnerable to stress as trafficking of new mitochondria and other cargo is impaired. The organelles likely not only provide energy but also calcium buffering and other protective measures. Once mitochondria are depleted, it becomes more likely that post-synaptic receptors will be lost and synapses disrupted. Considering that HD is probably a failure of synaptic function first, Zheng would be interested to know whether Htt plays a direct role in AMPA receptor trafficking. One possible scenario might be through Htt's interaction with cofilin, which is required at the site of vesicle fusion. The actin cytoskeleton forms not only a structural meshwork but also a dynamic "actin cortex" immediately under the intracellular side of the plasma membrane that plays an active role in endocytotic and exocytotic processes. Remodeling of this actin cortex requires dynamization by cofilin. Similar to the plastic remodeling at dendritic spines, growth cones require dynamic remodeling of the actin cytoskeleton and high levels of ATP. It has not yet been determined whether Htt plays a direct role in this process, but its interaction with cofilin might be relevant here as well.

How might Htt's role in intracellular transport differ with cell type? Why might some cells be more vulnerable?

This question may have more to do with the specific activities—and therefore energetic needs—of individual cell types rather that whether the Htt protein has different roles various cells. Medium spiny neurons (MSN) are more likely victims of circumstance rather than selectively vulnerable to mutant Htt *per se*. For example, if transport or other cellular processes are disrupted, MSNs may suffer in several ways: from loss of trophic support from cortical neurons, from disruption of mitochondrial availability at dendritic spines, and from loss of other transported cargoes required around the cell including neurotransmitter receptors. But HD is not a disease of cell death, it's a disease of cell signaling loss, and MSNs are highly active and plastic when it comes to signaling.

Further, Marc Hellerstein cautions, "It's important from a human therapeutics point of view to pay attention to cell selectivity," because subtle differences between

cellular conditions can be exploited. For example, cells express different isomers of tropomyosin, which affects cofilin binding and may be relevant to Htt function as well. Pal summed up by suggesting that mutant Htt may cause ubiquitous defects that have cell-type-specific biological consequences. Together, the conditions might result in what looks like selective cytotoxicity in striatal neurons. Finally, we might gain a better appreciation of real cell-specific differences by mining the literature or conducting new studies to measure Htt protein expression levels.

How would one validate any mechanism as therapeutically relevant for HD? What platforms and outcome measures would be required to show a perturbation of a particular mechanism would impact HD relevant outcomes? What criteria would one use to decide among targets for drug-discovery programs?

As with any disease—and neurodegenerative diseases in particular—it's difficult to determine what can be ruled as "cause" and what is "effect," and harder still to tease out cellular mechanisms of compensation. But even compensatory mechanisms can inform you about the presymptomatic conditions of a disease, says Hellerstein. Signs or symptoms may ultimately result when these adaptations fail. The workshop included lively discussion about whether you need to understand the mechanism of disease in order to treat it, which was also related to the dilemma about targeting genetic vs. small-molecule approaches. Two main possibilities arose: use a genetic screen for phenotype, or, as Kleckner put it, use "a different ideology" to understand the entire mechanism and try to disrupt it at specific points. If this is the strategy, Kleckner went on, "you don't want to hear my next question: if the protein does 40 thousand things, how do you expect to affect disease pathology by figuring out any given one?" And this brought us back to the issue of whether you can affect one function to interfere with pathology. Holzbaur offered an example from her experience with rescuing motor neurons in SOD1 mice. "We couldn't affect it with one, two, three pathways, but once you hit four pathways, we got synergy that could rescue the motor neuron's life." And although it may seem intuitively easier to decrease (or antagonize) a pathway, participants agreed that increasing activity by "inhibiting the inhibitor" is a powerful strategy as well. Finally, Holzbaur and others cautioned, "nobody has shown that just slowing traffic causes HD. We can modulate all these pathways and not be getting at the disease." Which brings us back to the need for a biomarker—a biological substance that changes predictably with disease progression—to indicate when pathology has been altered by a specific pathway. On the other hand, trafficking might provide a drug-screening tool aside from lending clues about Htt's cellular role.

So does HD have a single control point? "Every complex machine has a single on-off switch," as Truant put it, but shutting off WT Huntingtin protein may not be equivalent to shutting off disease. (In fact evidence suggests that knocking down all cellular Htt altogether has profound deleterious effects.) If this on-off switch approach can work for HD, it's likely to be a genetic approach to stop production of mutant Htt. Kiselyov, who thinks about drug development, has to be pragmatic. The

drawback to this genetic approach is, as he called it, dealing with the "ugly practicality" of delivering an agent safely, effectively, and preferably cheaply to a hugely diverse group of patients. Therefore, he will consider all options whether from a genetic or small-molecule approach, with or without understanding all the protein's roles. Kiselyov concluded from the discussions that tubulin or motor proteins might be his first target, as a sort of "hammer" approach. Though this approach may be disruptive because of tubulin's ubiquity and importance, he doesn't want to think of any molecule as "taboo" if it might yield practical outcomes. Truant echoed this sentiment, citing examples of other molecules that might have seemed "insane" to target but have yielded successful therapies.

And Kiselyov echoed the sentiment from other participants, particularly Hellerstein, that we need to move toward involving humans earlier in the process and looking for phenotype as indicators of disease perturbation. The classic paradigm in pharma has been to start with an i*n vitro* target, move to an *ex vivo* target, then to tissue, a proof-of-concept experiment *in vivo*, and finally to trials and the clinic. "That's changing," said Kiselyov. "The entry point to the package is getting more phenotypic."

Hellerstein has ideas about how to facilitate that process in HD by focusing on the "causal processes" in disease. "I can list a half dozen things," to measure to determine the physiological outputs that are the driving forces underlying the progression or reversal of disease, he says. "You have to jump into something in human at some point." Hellerstein suggests he would start with neuronal transport and trafficking, including measurements of the dynamic turnover rate in the organism of growth-factor release, and BDNF release in particular. He would look at organelle biogenesis, and its flipside, autophagosome activity. And apoptotic cascades, protein turnover—particularly of Huntingtin itself—and actin filament assembly and disassembly would all be processes that might yield good candidates.

How can we make the best use of the tools available and keep moving toward successful therapies that will target disease?

While the tools available to study the Huntingtin protein and Huntington's disease do not all reflect the physiological state in humans, each has its own utility. However, participants agree that we need to use the full-length Htt protein (rather than fragments) to understand its function in real cells, and particularly for protein-protein interaction studies. It appears clear now that with Htt's size, complexity, and potential mechanical properties, fragments may give incomplete or misleading information. But Signer pointed out that, despite the shortcoming of fragment animal models, they develop polyglutamine disease quickly; "whether that disease is HD or not we don't know." And *Drosophila* models will likely need to be used in preclinical development of any therapeutic drug.

Saudou's pARIS construct, a synthetic cDNA of the full-length human Huntingtin (Htt) sequence, will extend our ability to manipulate and examine the full-length protein in cells. The effort to create the construct arose from the difficulty in making specific modifications to the full-length protein and, as discussed at length, because

of problems with using fragments of the protein in animal models of HD. The construct is engineered to be insensitive to siRNA directed at four commonly targeted sites. This allows one to first knock down the endogenous Htt in cells and then re-express the human construct of the full-length Htt. Saudou wanted to allow for specific modifications to the molecule, including point mutations, fluorescent tagging, and internal deletions. To achieve this, they removed the normal restriction sites and introduced unique sites every 1 kB. These small fragments may then be mutagenized or tagged before re-insertion to the complete sequence. The construct has also been optimized for codon usage in various species.

Jim Wang, a CHDI consultant, is building a database that will allow CHDI to analyze data from hundreds of genes that might affect HD outcomes. Wang pointed out the benefits of the "manageable" fly model. Despite its removal from human disease, the *Drosophila* model may provide a good platform to examine some basic functions of Htt, with expression of the full-length protein. In flies, when you express mutant Htt with eye drivers, you see a phenotype of eye degeneration, and when you use a pan-neuronal driver, you get a fly with a deficit in a natural motor climbing response and a shorter lifespan. Interestingly, when you express full-length Htt compared to fragments, the phenotypes are significantly delayed. Although most of the data is from fragment expression, Wang presented a list of about 100 genetic modifiers, many of them related to synaptic proteins. This list of modifiers overlaps significantly with a list of Htt-associated proteins.

For further consideration

While the focus of HD research has been centered on neurons, the fact remains that Htt is expressed in other cells and even in other tissues. Saudou has seen effects of Htt disruption on ciliogenesis, which may affect developmental neuroblast migration because of impaired cerebral spinal fluid (CSF) circulation. Holzbaur and Kleckner agreed that further investigation of Htt's role in the nucleus is warranted, particularly its potential effects on chromatin and spindles. And Truant pointed out that insulin release is impaired with HD, leading to an impaired handling of glucose challenge in patients. This may represent a general defect in exocytosis in peripheral cells, mirroring the synaptic defects seen in neurons. "We need to step outside of the CNS-centric view," said Saudou.

Finally, Signer added this consideration: although HD relies on a single gene, other genetic factors may contribute to disease. Among HD patients with the same predicted age of onset, some genetic single-nucleotide polymorphisms (SNPs) can delay disease onset. Epigenetic factors and gene suppressors may also affect disease onset and progression.

Suggested Experiments

At the workshop's conclusion, participants were asked to propose experiment; these are summarized here. Other Open Questions and Action Items are included at the end of this section.

- Ramee Lee believes that when it comes to Huntingtin and its associated proteins involved in protein trafficking, "all the basic players have been identified." She would like to know more about the effects of post-translational modifications on protein function and ultimately on traffic control.
- Randy Singh wants to find out how poly-Q length affects the function of Huntingtin (Htt), because that mutation underlies the difference between a functionally normal protein and a diseased one. He would also like to see how post-translational modifications interact differently depending on poly-Q length.
- Arun Pal points out that we need a "comprehensive kind of description for HD." We're now living in the age of high-content analysis, what we need is a "multi-parametric signature" of the disease with less focus on specific pathways. He suggests that a panel of morphological descriptors could be used as a screening assay, even if the molecular players and pathways have not been named. The assay could be used both as a "rescue readout" and could be used in combination with bioinformatics to predict any missing molecular players. AP also brings up the recent shift in focus in HD research from aggregate toxicity to other aspects of disease. Pal would like to be sure that aggregates are not the problem.
- Marc Hellerstein agrees that we need a more "systems approach." He suggests using labeling tools (which entail feeding an animal or human heavy water [D_2O]) to look at animal models of HD with varying poly-Q length. He would investigate which pathways have been altered by the mutation and which ones correlate best with a pathological phenotype of disease. His sense is that the search would reveal neuron-specific pathways that regulate cargo, including mitochondria, turnover of proteins like actin, cofilin, profilin, and Huntingtin itself. Apoptotic pathways, neurotrophic factor transport, and neurotransmitter turnover might also be altered. Once you have established reliable readouts, you could use this tool to assess various interventions *in vivo*.
- Patrizia Fanara suggests that the critical factor of the KineMed labeling technique is the priority you place on each pathway. With access to transgenic HD mice, she says she would look for a brain chemistry signature from neurons and other cell types to identify real biomarkers. She also points out that we may benefit from starting in human HD patients and applying lessons learned back to the animal models. Information gained about the disease process from this signature in humans could help guide research at the basic science level.
- Ray Truant finds value in looking at small molecules that modulate these pathways in one direction or the other. He cautions against having "prejudice" about what pathways might be valuable targets, and says that even essential cellular functions can sometimes be successful targets. His immediate intent is to look at these phenotypes *ex vivo* in peripheral cells from human HD patients, and then to go back to a model organism.

- Fred Saudou appreciates the shift from looking only at aggregation to careful consideration of the natural function of full-length Huntingtin in cells. He wants a better understanding of how such a complex disorder can arise from these transport deficits and believes we need to move away from a "pure CNS view" to look at other tissues. In terms of experiments, he would like to visualize transport of BDNF in neurons and to perhaps explore Holzbaur's methods of force measurements in Htt-associated transport.
- Shihua Li is working on producing a larger animal model of HD as an intermediate between mouse and human, somewhere in the range of 20 lbs, created ideally as a knock-in mutation. She raises the point that the best target would be mutant Htt itself.
- Jim Wang expressed his fondness for the *Drosophila* model: it's manageable and some behavioral phenotypes in the fly could be good assays for Htt transport manipulations. He suggests systematically knocking down the genes that we know are important in cellular transport and examining them in these fly phenotypes. (See list of genes from Wang.)
- Jim Zheng would focus on the area of mRNA translocation and localization in neurons. This complex process depends on timing, mechanics, and activity-dependence, all of which determine whether proteins are properly expressed in the cell. Proteins could include neurotransmitter receptors and trophic factors, so defects would have wide-ranging effects.
- Jamshid Arjomand has the task of creating assays or readouts using HD stem cells, and he wants to make sure that transport defects really do have pathological disease effects. His proposal for Saudou would be to make a mouse with the Serine 421A or D knock-in mouse to show that phosphorylation at that site not only helps trafficking but also that it improves some behavioral phenotype. (Saudou responds that they actually have that mouse on a wild-type background, and that it appears grossly normal. Next they will try to cross these mice with an HD model mouse.)
- Folma Buss would like to cross myosin VI knockout mice with the best HD model mouse. This interaction may represent a targetable transport pathway for HD pathology, because optineurin is an Htt binding partner, and myosin VI enhances optineurin. She would request guidance in choosing the best mouse with a light HD phenotype.
- Jim Bamburg wants to look at rods. Ultimately, he'd like to use a knock-in mouse that expresses a mutant form of cofilin that can't be incorporated into rods, a so-called rodless mutant, and cross these mice with models of HD. More immediately, he'd like to use a single viral vector to knockdown and replace wildtype cofilin with rodless cofilin. These experiments could be done in an organotypic slice from an HD mouse; he would look for rescue of defects in rodless cells.
- Erika Holzbaur could answer many questions about the proposed hypotheses for Huntingtin's role in cytoskeletal dynamics using "reductionist assays" with purified Htt (as they have done with purified motor proteins) and minimal

filament systems. The next step up would be to use her lab's system of reconstituted Huntingtin-dependent bidirectional vesicular transport and look at elements like force generated and the specific motor proteins involved. Third, she believes that considering we don't know everything about dynein's role in cytoskeletal dynamics, we don't know everything about Htt either.

OPEN QUESTIONS/ ACTION ITEMS

- Holzbaur: what are the actual specific forces (in the pN range) that HEAT repeat regions require—not to deform the molecule, but to achieve these mechanical properties? (Discussion suggests that thermal forces are sufficient, but actual physical measurements may be valuable.)
- Arjomand adds a request for an experiment for Truant: Use William Yang's N-terminal double-phosphorylated Serine 13 and 16 phosphomimetics, which don't display an HD-like phenotype in mice. In these animals, do cells form nuclear rods when heat shocked or not? Truant responds: they're working on that question, but they haven't looked yet. When they do, they'll be using real phosphorylated protein, not phosphomimetics.
- Wang wants to know: in the human neural stem cells in which transport was rescued by knock-down of mutant Htt, what would happen if you knocked down both mutant and wildtype Htt? Saudou says has not looked at this but says he can do it. He predicts that loss of wildtype Htt will look similar to expression of mutant, with this reduction in transport. CHDI is pursuing a therapeutic strategy whereby wildtype Htt is reduced, so it would be beneficial to know the results of this experiment. Wang predicts that either 1) it won't work and may make patients worse, or 2) it will work, meaning the Htt role in transport isn't critical to rescue the disease pathology.
- Cross-check Hellerstein's list of approximately 65 protein kinetics candidates with Martin MacIntosh's published list of genes affected by HD.
- Incidentally: According to Pal, immortalized striatal cell lines from the MacDonald lab are not suitable for studying membrane trafficking in the context of HD. The cells lines require differentiation into post-mitotic neuron-like cells, and the time course of cell development (and death) does not allow for meaningful inquiry about trafficking. The cells do not show any processive movement of vesicles over long distances in the neurites. To understand pathological biology, primary neurons are preferred.
- Ethan Signer would like Saudou to systematically examine any functional differences between cells expressing fluorescent tags on the N- vs. the C-terminus. Some anecdotal evidence suggests that the tag location may have functional ramifications, particularly for subcellular localization. In general, Saudou's assessment is that tags have not interfered with any cellular function that they have measured. After siRNA of a cell's endogenous Htt, re-expression of the WT pARIS construct of Htt restores organelle and transport functions. These

are not restored with expression of the mutant Htt protein. Importantly, this suggests that the tags do not interfere with the mutant's toxicity per se.

- Ray Truant has undertaken a study of 14 cell characteristics that he will systematically examine in cells null for Htt. When a GFP-labeled WT Htt protein is expressed, which specific functions are restored? The next step will be a two-color system, with one for wildtype and one for mutant Htt. The results of these studies will be placed in an online repository.
- There was discussion about the importance of having at least some idea about the protein expression levels in the cells that you're using for experiments. Shihua Li has compared both protein and mRNA level of Htt in various lines, including R62, N171, Yak, and Bac. In some lines, low protein levels did not match the high transcript levels. (References: Li, S-H., Schilling, G,. Young, et al. Huntington's disease gene (IT-15) is widely expressed in human and rat Tissues. Neuron 11: 985-993, 1993. Sharp, A.H., Loev, S.J. Schilling, G., et al. Widespread expression of Huntington's disease gene (IT-15) protein product. Neuron 14: 1065-1074, 1995.) Protein can also be measured with a FRET assay made by Novartis. The consensus among participants is that reviewers aren't taking the issue seriously enough and that it likely affects data. A related concern is that studies typically use wildtype animals as controls for HD mouse models, particularly those using protein fragments. For example, the real control for an R62 mouse should be a counterpart mouse with a short-poly-Q region inserted into the same site in a fragment.
- Jim Bamburg would like to create a "rodless mutant" mouse that expresses a form of cofilin that does not form rods but retains 90% of its function in terms of actin dynamics.
- Evidence from several labs suggests that a therapeutic strategy to completely knock out both mutant and wildtype Htt in human patients may not bear fruit, because cells seem to need a basal level of wildtype Htt. This issue may need further investigation. According to Truant, mouse embryonic fibroblasts after complete Htt knock-down display a very sick phenotype, particularly in ER morphology, and don't divide. Marcy MacDonald has reported that loss of more than roughly 50% of wildtype Htt results in an inviable mouse. However, others have reported that Htt-null ES cells grow well in culture.