Autophagy in the Pathogenesis and Treatment of Huntington's Disease

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Abstract

Recent studies suggest that enhancing the clearance of mutant huntingtin could help alleviate the cellular pathology underlying Huntington's disease (HD). To examine this possibility, the Hereditary Disease Foundation brought together leaders in the field of autophagy to discuss the role of this process, as well as other clearance systems, in the degradation of mutant huntingtin and its various aggregated forms. Examining both published and unpublished data, participants identified gaps in our knowledge that could hamper the development of clearance-based therapies, and suggested approaches to help fill them in. In addition, they discussed new ways to boost the clearance of huntingtin toxic species and their associated limitations and potential side-effects.

Key issues to emerge included the need for investigating in greater depth autophagy's regulatory mechanisms, the kinetics of huntingtin clearance, mutant huntingtin's ability to disrupt cellular clearance mechanisms, and wildtype huntingtin's role in autophagy. Various potential links between the clearance systems and huntingtin emerged (e.g., huntingtin being both a substrate for autophagy, as well as part of the autophagic machinery), highlighting the complexity of the problem. Nevertheless, participants were optimistic about the future, noting that the workshop helped pinpoint key issues to address, as well as opened new communication channels and collaborations that promise to accelerate the development of HD therapies.

Introduction

HD is a complex disease that has challenged researchers with its multiple facets. Huntingtin can interact with over 150 protein partners and, when mutated, interferes directly and indirectly with dozens of cellular pathways. Thus, it has been difficult to develop therapies that effectively target HD's toxic effects.

An appealing alternative is to simply eliminate the mutant protein. As noted by Kimberley Kegel, the approach bypasses the need to dissect the complex ways in which mutant huntingtin mediates toxicity. Several efforts to do this are now underway, including the use of exogenous molecules, such as intrabodies and RNAi, to block mutant huntingtin production or activity.

But it may also be possible to harness the cell's own clearance mechanisms. As noted by Carl Johnson, all long-lived organisms are affected by the accumulation of protein aggregates so it is reasonable to expect that evolution has developed mechanisms to de-toxify them. Discussing various ways in which cells clear unruly proteins, participants at the workshop examined new ways to tackle HD.

An inspirational account

The optimism and desire to speed therapeutic research was fueled by Carlos Urrutia and his wife who generously shared their experiences coping with HD. Thirteen years ago, the couple began noticing in Carlos the first symptoms of HD—the disease that consumed Urrutia's mother's physical, as well as mental, health and eventually killed her. With exceptional strength and optimism, the couple has been adapting to life with HD and its consequences. Perhaps partly because of their positive outlook and pro-active behavior, the progression of Carlos's disease has been remarkably slow. But still they face innumerable challenges: dealing with the negative reactions from Carlos's family, managing everyday life with two teenage children, coping with the memories of Carlos's mother who became increasingly violent and abusive as HD progressed. The couple provided participants with a picture of life with HD and its many

associated problems, as well as information of potential scientific relevance (e.g., the anticoagulant coumadin's apparently positive effects on Carlos's speech and cognition). More importantly, Carlos and his wife inspired participants to find new ways of understanding HD and curing it.

Huntingtin and the ubiquitin proteasome system (UPS)

Under most conditions, the UPS is the major pathway for degrading intracellular proteins. It is thus likely—although not proven, as noted by Carl Johnson—that monomeric huntingtin is normally processed through this pathway. Recent studies indicate that eukaryotic proteasomes, however, are limited in their ability to degrade polyglutamine proteins. As summarized by Ron Kopito, Fred Goldberg's team found that yeast and mammalian, although not archaeal, proteasomes are unable to cut polyglutamine stretches 10-30 residues long *in vitro*. Goldberg proposes that, when degrading polyglutamine proteins, eukaryotic proteasomes must release polyglutamine-containing fragments for further hydrolysis by other peptidases.

Kopito noted, however, that the mammalian experiments were performed using proteasomes treated with detergent which releases the 19S and 20S accessory proteins normally associated with the proteasome. This is potentially problematic because the 19S protein recognizes ubiquitinated residues and helps unfold and feed substrates to the proteasome, activities which are key for proteasome processivity. The physiological significance of the yeast experiments is also limited because of the use of yeast mutants that have a constitutively open pore. Kopito noted that other experiments, performed under more physiological conditions, however, support the findings. Using a lysate optimized for UPS activity which includes proteasome accessory proteins, other researchers have found that the polyglutamine regions of in vitro-synthesized proteins are not cleaved well by eukaryotic proteasomes.

The fate of polyglutamine fragments released from the proteasome is unknown, but a compelling scenario was presented by Eric Reits. Reits explained that intracellular aminopeptidases degrade small peptides released by the proteasome within seconds, almost regardless of amino acid sequence. Peptides longer than 15 amino acids, however, must first be cleaved by the peptidase TPPII, which has both exo- and endopeptidase activity. Thus, polyglutamine stretches released by the proteasome are likely degraded by TPPII.

Using a fluorescence quenching approach to monitor cleavage of a construct comprising two undegradable sequences separated by a polyglutamine linker, Reits has found that TPPII is indeed capable of degrading polyglutamine. However, it is very inefficient. Under normal conditions, the inefficiency is probably not a problem because non-expanded polyglutamine fragments are expected to require a single clip from TPPII to become substrates that are small enough to be degraded by other peptidases. When proteins contain expanded polyglutamine stretches, however, long polyglutamine fragments may start accumulating before TPPII can cleave them sufficiently to become substrates for other proteases, and this may promote aggregation.

Goldberg has suggested that polyglutamine chains exceeding the lengths of normal proteasome products (2-25 residues) may fail to exit the proteasome and interfere with its function. In addition, *in vitro* and *in vivo* data from Richard Morimoto's lab suggest that mutant huntingtin and other expanded polyglutamine proteins are kinetically trapped within the proteasome as partially degraded substrates. Some of the partially degraded products are probably released from the proteasome. Reits agrees with this possibility and suspects that

proteasome blockage is only partial such that large protein fragments are falling off semi-clogged proteasomes.

As expanded fragments accumulate, they start to oligomerize. Indeed, when Reits overexpresses TPPII in cells with expanded polyglutamine proteins, the number of visible aggregates decreases. Conversely, if he inhibits or knocks down TPPII with RNAi, he sees an increase in aggregates. The increase is similar to that observed when he blocks proteasome function. Interestingly, the effects of TPPII inhibition on aggregate formation are reversible, suggesting that TPPII activity might be particularly important for preventing the early stages of protein aggregation.

The idea that the efficiency of degradation might be key for determining aggregate formation is also supported by experiments from Nico Dantuma's lab, as noted by Kopito. Dantuma reported that speeding up targeting to the proteasome by introducing an N-end rule degradation signal in an expanded polylgutamine protein reduced the incidence of nuclear aggregates and cellular toxicity, presumably by accelerating the clearance of soluble substrate.

Not surprisingly, several groups have reported an increase in aggregates when proteasomes are inhibited. Paul Taylor and David Rubinsztein, for example, noted they have observed this in different systems. Taylor noted that the off-rate and half-life of the inhibitor is key. Using inhibitors such as lactacystin, Taylor described a temporary effect which contrasted with the long-term increase in aggregate formation and eventual cell death caused by inhibitors that are longer lived and bind more permanently to the proteasome. Rubinsztein described seeing an increase in soluble mutant exon 1 levels using lactacystin or epoxomicin, a more specific proteasome inhibitor. However, while aggregation increased with lactacystin, it decreased with epoxomycin. Ai Yamamoto and Kopito suggested this might be due to an increase in chaperone function induced by a stress response to the strong inactivation induced by epoxomycin. Alternatively, Reits proposed, lactacystin, but not epoxomycin, might be inhibiting the proteasome only partially, thus promoting the production of fragments that act as nucleators of aggregation.

The inability of the UPS, and possibly the TPPII protease, to effectively clear mutant huntingtin may result in a vicious cycle. Inefficient clearance of mutant huntingtin may promote the formation of aggregates and, as previously shown by Kopito, aggregates dramatically interfere with proteasome function. Although it is still unclear how early in the disease process the UPS is affected, Kopito noted that it is unquestionably dysfunctional. Indeed, there is a massive accumulation of ubiquitinated proteins. Ray Truant asked whether endoplasmic reticulum stress, which has been implicated in the pathogenesis of several conformational diseases including HD, could also be affecting UPS function. A recent paper by Menendez-Benito and colleagues indicates that, indeed, ER stress has a general inhibitory effect on the UPS.

Marian DiFiglia cautioned, however, that, studies in both HD mice and patients on proteasome function have yielded contradicting results. One complicating factor is that proteasome activity appears to naturally decline with aging. Another consideration, pointed out by Yamamoto, is that proteasome inhibition may not be uniform within neurons. For example, if inhibition occurs predominantly at synapses, it may be difficult to detect using approaches that do not distinguish between subcellular compartments. A recent study by Kopito and colleagues, however, indicates that formation of protein aggregates targeted to either the nucleus or the cytoplasm results in global impairment of UPS activity in both cellular compartments. Because impairment appears to occur in compartments that do not have aggregates or aggregate-prone

proteins, and because aggregates do not interfere with core proteasome function *in vitro*, the authors proposed that aggregates probably do not cause direct choking of proteasomes.

Thus, the emerging picture of the UPS's interaction with mutant huntingtin is complex: the UPS appears to be important for effective clearance of huntingtin, but it might also be the source of toxic fragments that nucleate aggregates which, in turn, disrupt UPS function. In addition, ER stress induced by mutant huntingtin may contribute to UPS dysfunction. Another important observation is that dysfunction of the UPS caused by mutant huntingtin may have important effects on another clearance mechanism, autophagy (see below). Taylor and Eric Baehrecke have found that genetically blocking proteasome function in fly eyes results in a dramatic increase in autophagic vacuoles at all developmental stages. Knocking down the autophagic gene ATG_{12} enhances the phenotype and treating flies with rapamycin, which activates autophagy, ameliorates the phenotype. Similar results are obtained when proteasome function is disrupted by a mutant androgen receptor with an expanded polyglutamine stretch in a fly model of SBMA.

Action items

1. Clarify how the UPS is involved in wildtype and mutant huntingtin degradation. Despite the data discussed above, it is still uncertain to what extent the UPS plays a role in huntingtin degradation. Several participants noted that the full-length protein has a 3.5 to 4 day half-life which, as noted by Kopito, seems inconsistent with UPS degradation. One possibility, proposed by Ana Maria Cuervo, is that huntingtin is released slowly from membranes, making it only gradually available for proteasome degradation. Participants also discussed the importance of defining the role of the UPS, as well as of other degradation pathways, in the clearance of huntingtin fragments (see *The challenge of monitoring the fates of huntingtin's multiple species* below).
2. Examine the role of TPII activity in huntingtin clearance in greater detail. As noted by Reits, TPII may be relevant for the early steps of huntingtin oligomerization. Thus, understanding its regulation could be of therapeutic importance. Reits's team is currently working on characterizing TPII—not an easy task given the protein's complex structure which includes two intertwined strands each composed of 11 monomers.
3. Clarify the state of UPS function in HD models. To confirm that UPS function is disrupted in HD models *in vivo* and extend the understanding of this phenomenon, Kopito and a team of collaborators plan to cross R6/2 mice with mice expressing a UPS reporter.
4. Investigate the global consequences of UPS disruption and compare to HD alterations. Kopito's team is conducting proteomic analyses to obtain a profile of the proteins that accumulate when the UPS is inhibited. So far, they have identified a couple hundred proteins and find significant overlap with the profile of cells expressing mutant huntingtin.
5. Consider using new derivatives of epoxomycin in future experiments. Kopito noted that Craig Crew has developed epoxomycin derivatives that target more of the proteasome's proteolytic activities.

Autophagy and HD

Autophagy is a process of intracellular bulk degradation mediated by lysosomal enzymes. As noted by Rubinsztein, whereas individual proteins are most often degraded by the UPS, autophagy is recruited to degrade multi-protein structures, including protein aggregates. Cuervo explained that there are three recognized forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). During macroautophagy, double-membrane sequestering vesicles, or autophagosomes, fuse with lysosomes. During microautophagy, cytosolic components are directly transferred into the lysosome by invagination of the lysosomal membrane. And during CMA, specific cytosolic proteins are translocated across the lysosomal membrane via the lamp2A receptor on the lysosome.

One of the first indications of a link between autophagy and HD was provided by Kegel and DiFiglia who observed huntingtin-labeled vacuoles with ultrastructural features of early and late macroautophagosomes in HD neurons. Early in the disease process, the researchers observed multivesicular bodies and a proliferation of endosomal and lysosomal bodies in human HD brains, particularly in cortical neurons.

Offering descriptions of their most recent electron microscope observations, DiFiglia discussed data obtained from cell expressing the first third of mutant huntingtin. At the core of the huntingtin-associated structures, the researchers see cathepsin D, whose activity has been shown to increase in response to huntingtin expression in cultured cells, and a fibrillar form of huntingtin, labelled by the EM48 antibody, intermixed with stacks of membranous sacs. A ring of soluble huntingtin, which can be digested away with proteinase K and depends on prolines adjacent to the polyglutamine region of huntingtin for its formation, surrounds the core. DiFiglia noted that the ring might be an oligomeric structure because it appears as beads-on-a-string. She also pointed out that many proteins appear to be sucked into this region, including vesicle-trafficking proteins and some chaperones. Interestingly, the proteasome, heat shock proteins, and ubiquitin are segregated into distinct locations. It is not clear if the entire structure is surrounded by a membrane.

The formation process seems to start peripherally, and then move to the perinuclear region, forming labyrinthine shapes. DiFiglia has observed membranes surrounding huntingtin with smaller beaded chains, even before aggregates are detected. These structures, she suggested, may be precursors of the bodies described above, which are larger and more heterogenous. An alternative explanation, proposed by Cuervo, is that two different processes are unfolding that are not necessarily sequential. The smaller structures might be associated with microautophagy, rather than macroautophagy.

Using light microscopy, other participants have also observed autophagic-like structures. Kopito and Yamamoto, for example, described perinuclear LC3-positive doughnuts associated with huntingtin aggregates (during autophagosome formation, LC3 is lipidated to form LC3-II, which is used as a marker of macroautophagy). Kopito added that LC3 is not simply trapped in the aggregate because it remains soluble and other autophagosomal markers, including ATG 5, 12, and 16, are also present.

There is also pharmacological evidence supporting an important connection between autophagy and HD. DiFiglia found that blocking macroautophagy in cells expressing amino terminal huntingtin fragments increases fragment levels and aggregates, and reduces cell viability. In support of these findings, Rubinsztein and colleagues have observed an accumulation of tagged mutant exon 1 when cells are treated with inhibitors of various stages of

the macroautophagy pathway. Moreover, rapamycin enhances the clearance of the mutant protein, protects against neurodegeneration in a fly model of HD, and improves behavioral symptoms in N171 mice (see *Treatment candidates* under **Therapeutic Directions**).

Genetic screens have also implicated autophagy. Using microarrays to identify genes that are upregulated in cells expressing polyglutamine proteins, followed by siRNA screens to assess the effects of knocking down these genes, Yamamoto identified several autophagy genes that seem to be required for aggregate clearance, including lysosomal membrane proteins lamp1 and 2. She has also found that cells that overexpress beclin-1, a component of the mammalian autophagy system, clear huntingtin more effectively. Johnson added that other genetic screens have identified HD modifiers associated with autophagy.

Open Questions

<u>*Atypical autophagy in HD?*</u>

There are still many uncertainties about autophagy´s relationship to HD, however. Indeed, it is still unclear whether the vacuoles associated with huntingtin are *bona fide* autophagosomes. Kopito noted that whereas autophagosomes induced by the cystic fibrosis transmembrane conductance factor (CFTR) are stereotypical, the structures induced by huntingtin expression are often not. Sulzer agreed, adding that his group has observed huntingtin-associated autophagosome-like structures far out in axons. Although they stain with MDC—a classic, albeit non-specific autophagic marker—they likely do not fuse with lysosomes, which are scarce in axons, until they move into the cell body. In addition, DiFiglia sees tubulation of endosomal membranes associated with both wildtype and mutant huntingtin expression.

The slowness of the process is also disconcerting. Yamamoto, who is beginning to study how huntingtin autophagosome-like structures evolve over time, noted that she observes extremely long-lived LC3-positive rings. Baehrecke proposed that the rate-limiting step in huntingtin autophagy may be the formation and recruitment of pre-autophagosomal structures to aggregates, but once this pre-autophagy processing is completed, autophagy proceeds at its normal rate.

One problem with current light and electron microscopic data is that overexpression of mutant huntingtin may result in some artefactual alterations. Inclusion formation, for example, is critically dependent on huntingtin concentration, in particular relative to chaperone concentration, noted Kopito. DiFiglia agreed that overexpression is potentially problematic, but emphasized that her group has seen a clear autophagic response in HD post-mortem tissue, including the presence of multivesicular bodies and the proliferation of lysosomes and endosomes. In addition, Yamamoto pointed out that different cell types, expressing inducible mutant huntingtin constructs at different levels, all seem to clear huntingtin aggregates within 5 or 6 days suggesting that aggregate clearance mechanisms do not vary significantly with mutant huntingtin levels.

Another problem is the paucity of quantitative results. As noted by Cuervo, knowledge of the relative abundances of the different structures observed could provide clues to the significance of each structure, as well as shed light on how the recruitment of different clearance pathways changes over time.

Cuervo also noted that the observed peculiarities might simply represent physiological variants of autophagy which haven't been described yet. For example, she pointed out that

autophagosomes induced by viral infection are larger, take longer to form, have different cytoskeletal requirements, and respond differently to 3-methyl adenine than other autophagosomes. Baehrecke added that his group has seen structures associated with cell death in mammalian cells that look like autophagosomes but do not contain LC3. Some researchers, as noted by Rubinsztein, object to the classification of these structures as autophagosomes. Yet Kopito argued that using a single marker to decide whether a structure is an autophagosome or not is too limited. He added that it wouldn't be surprising if the autophagic degradation of huntingtin aggregates differs significantly from other types of autophagy.

Rubinsztein suggested using an operational definition of autophagy, noting that static measurements and images of structures can be misleading and difficult to interpret. He considered that the term autophagy should be restricted to processes in which the clearance of an autophagy substrate can be measured and inhibited by specific drugs, such as 3-methyl adenine. However, Kopito argued that not enough is known about autophagy to limit its definition by drugs or other individual markers. Even in the best studied systems, such as yeast, many open questions remain. Thus, Kopito urged participants to avoid the historical baggage of most current definitions of autophagy and simply use descriptive terms to discuss their data.

Does HD cause autophagy blockage or induction?

Another fundamental question, raised by John LeMaster and Randy Nixon, is whether the observed increase in autophagosomes associated with mutant huntingtin is a sign of autophagic induction, or the result of autophagic blockage. Rubinsztein noted that cells with mutant huntingtin, have more autophagosomes, more LC3 II, and decreased mTOR activity, all of which are consistent with the induction of autophagy. His studies indicate that mTOR is sequestered in mutant huntingtin aggregates, such that soluble mTOR levels are reduced and mTOR phosphorylation of its substrates S6K1 and 4EBP1 is impaired. LeMaster agreed, adding that genetic manipulations also support this idea. In fact, as noted by Yamamoto, autophagy seems to be maximally induced by polyglutamine protein expression given that starvation, which induces autophagy, is unable to further increase the autophagic clearance of long-lived proteins. (A caveat, however, is that one of the proteins being measured in this experiment is the over-expressed polyglutamine protein itself).

Huntingtin-mediated induction of autophagy does not preclude the possibility that mutant huntingtin also disrupts or blocks autophagy, as noted by Zsolt Talloczy. Indeed, the slow rate of huntingtin clearance and the persistence of autophagosome structures described above, are consistent with this possibility. Yamamoto and Kegel suggested mutant huntingtin might impair autophagosome-lysosome fusion. Yamamoto added she has observed partial blockage of autophagy even when expressing exon 1 with a wildtype polyglutamine stretch. However, Rubinsztein doubted that fusion is importantly affected, at least before inclusion formation. If fusion is blocked, he argued, then stimulation of autophagy should be incapable of enhancing clearance, yet he observes rapamycin improving clearance. In addition, his group recently observed that mutating the dynein machinery, which impairs autophagosome-lysosome fusion, enhances the toxicity of mutant huntingtin. As noted by Cuervo, it is possible that disease alterations change over time (see *A multitude of factors affect clearance mechanisms in HD)*. For example, fusion alterations may be overcome initially, but cause problems later on. Rubinsztein agreed noting that, although mutant huntingtin does not seem to directly affect dynein function, it interferes with kinesin function which, in the long run, might result in dynein alterations given these motors' complementary roles.

Regardless of the mechanisms, clearance deficiences appear to be reversible. Studies of Yamamoto's inducible mouse model of HD indicate that huntingtin aggregates can be cleared if huntingtin expression is turned off. Yamamoto proposed that the kinetics of aggregate formation and clearance probably play a key role: the two processes appear to be competing—when aggregate formation is shut off, clearance can catch up. In this view, as pointed out by LeMaster, the key problem is not an overall inability to clear mutant huntingtin, but an inability to do so efficiently. Participants noted that it will be important to investigate how the clearance of other, non-huntingtin, proteins are affected in HD.

A normal role for huntingtin in autophagy?

A major way in which mutant huntingtin may disrupt autophagy is by failing to perform its normal functions. As noted by Kegel and Truant, several observations indicate that wildtype huntingtin may play a role in autophagy. Kegel noted that huntingtin interacts directly with membranes and with proteins involved in vesicle transport. In addition, Truant noted that huntingtin's first 17 amino acids are extremely conserved and form an amphipathic alpha-helix which targets huntingtin to vesicles. The 17 amino acids alone localize to the endoplasmic reticulum, but adding more huntingtin sequence, including the region Kegel identified as binding to membranes, results in targeting to late endosomes and autophagic vacuoles. Using overlay assays, Kegel sees binding of huntingtin fragments that include exon 1 to autophagic vesicles.

Kegel noted that there are direct and indirect ways in which huntingtin may be involved in autophagy. One possibility is that huntingtin is directly involved in autophagosome generation acting at the surface of maturing vesicles. Thus, when huntingtin is mutated and clumps together, aggregates are found on the surface of autophagosomes. It is also possible that huntingtin affects autophagy indirectly through its involvement in neurite transport. When huntingtin is mutated, it may interfere with the distribution of autophagic components, not only by clogging neurites when it aggregates, but by failing to perform its normal transport functions.

Truant noted that serine phosphorylation seems to be importantly involved in regulating huntingtin's location. When a highly conserved serine is phosphorylated, huntingtin pops off vesicles and goes into the nucleus. Truant's studies using fluorescence recovery after photobleaching (FRAP) indicate that huntingtin is normally moving in and out of the nucleus. If the serine is mutated, the distribution of autophagic vacuoles changes dramatically. Truant also pointed out that the huntingtin interacting protein HIP-1 has been shown to act as a nuclear hormone co-receptor. Truant thinks that huntingtin is involved in the trafficking of vesicles from distant locations in the cell towards the nucleus.

Interestingly, polyglutamine by itself localizes to the nucleus and, at least for ataxin-1, its expansion results in the protein residing continuously in the nucleus. The polyglutamine region might also have effects on subcellular localization because it separates two vesicle-targeting domains, noted Truant. Whether and how changes in the length of the polyglutamine region affect the interaction of these domains, remains to be determined.

An important test to assess wildtype huntingtin's role in autophagy is to examine how autophagy is affected when huntingtin is knocked out. This test has not been performed yet, but participants discussed some relevant observations. For example, studies from Marcy McDonald's group indicate that embryonic patterning and morphogenesis are disrupted in huntingtin-deficient mice, and DiFiglia noted there is a sharp rise in huntingtin during early postnatal development. Interestingly, as noted by Yamamoto and Cuervo, autophagy is particularly important during development, particularly in the brain.

Does autophagy contribute to the generation of toxic fragments?

As discussed for the UPS, it is possible that autophagy contributes to the creation of mutant huntingtin fragments that are toxic and difficult to clear. DiFiglia noted that activation of autophagy by huntingtin increases cathepsin D activity which may help generate toxic fragments. At the 2004 CAG Boston Meeting, Yvon Trottier reported that cathepsin D co-localizes with huntingtin and, in vitro, can accelerate the generation of toxic fragments Cp-A and Cp-B. The acceleration, however, is only observed at neutral pH, suggesting that limited and local delivery of cathepsin D into the cytoplasm could result in huntingtin cleavage and the release of fragments that tend to aggregate. Other endo-peptidases may also be involved, since cathepsin D-deficient cells generate Cp-A, albeit in lesser amounts.

Moreover, DiFiglia's data suggest that autophagy may stimulate caspase-3 dependent cleavage of huntingtin and produce N-terminal fragments that accumulate as huntingtin bodies. Her group has found N-terminal fragments consistent with the size produced by caspase-3 cleavage in the cortex, striatum, and cerebellum of normal and adult onset HD brain. In addition, the expression of either wildtype or mutant huntingtin in clonal striatal cell lines increases caspase-3 activity. Caspase-3 has been considered a cell death caspase but, as noted by Baehrecke, it has been recently implicated in several normal cellular functions.

How autophagy may regulate caspase-3 activity is not clear. Baehrecke pointed out that autophagic cell death, although not autophagy induced by starvation, involves the activation of caspases. However, at least in flies, knocking out caspases does not prevent cell death nor autophagy, suggesting that autophagy and caspase activation are controlled by parallel pathways. Indeed, there is no evidence for cross-talk between these pathways.

Despite the apparent involvement of caspase-3 in HD observed by DiFiglia, observations from Cheryl Wellington in Michael Hayden's lab indicate that caspase-6, but not –2 or –3, is critical for mediating mutant huntingtin toxicity, noted Johnson. Wellington found that mutating huntingtin's caspase-6 sites is protective as revealed by decreased loss of cells in the striata of YAC128 mice.

The challenge of monitoring the fates of huntingtin's multiple species

One of the most important unresolved issues, as pointed out by Sulzer, is determining the clearance pathways used by different huntingtin fragments. Several studies indicate that endogenous, wildtype huntingtin has a 3.5-4 day half-life. But as noted by Kegel, the half-lives of huntingtin fragments are variable and mostly unknown. Kopito, who reviewed recent articles published on huntingtin clearance, noted that many studies don't include half-life measurements, and those that do, don't agree even on such a fundamental issue as the effect of polyglutamine expansion on huntingtin half-life—some report large differences between mutant and wildtype proteins, while others find none.

There are several reasons why monitoring huntingtin and its fragments has proven so challenging. As noted by Kopito, a classic approach to track protein turnover is to perform radiolabel pulse-chase experiments followed by immunoprecipitation to identify specific proteins. One problem with this approach, however, is that a large number of fragments are generated that are very difficult to consistently distinguish on Western blots, noted Kegel. In addition, fragments can become undetectable when they lose the antigenic regions of the particular antibodies being used. Not surprisingly, differences in the choice of antibodies have made comparisons between studies very difficult. Furthermore, several studies have relied on

antibodies that bind to the N-terminal of huntingtin, but do not distinguish between mutant and wildtype proteins, making it difficult to discern the origins of fragments. Moreover, wildtype huntingtin's very long half-life makes it difficult to monitor over several days.

Participants noted there are other techniques to study protein turnover, including the use of fluorescent tags, promoter shut-off, use of cycloheximide, and photoactivation approaches. However, all these tools have limitations as well. A major problem, not easily circumvented by any technique, is mutant huntingtin's tendency to aggregate. As summarized at a previous workshop, the ability of N-terminal fragments to re-assemble into dimers or higher order oligomers during electrophoresis is a source of altered mobility and variability, especially in the case of material derived from inclusions which are initially resistant to SDS solubilization. During electrophoresis and in the absence of significant heat, re-folding and re-formation of aggregates or oligomers may occur even in the presence of SDS. Not only is the migration of different huntingtin species affected, but the availability of their epitopes can change.

While the artificial oligomers formed in vitro are a technical nuisance, the physiological oligomers that occur *in vivo* are important species to monitor in their own right. Johnson noted that most studies to date have focused on either soluble huntingtin monomers or mature inclusions, but intermediate oligomeric states have been relatively neglected. One problem, noted Kopito, is that aggregates are difficult to define experimentally. Most studies use solubility as an assay, but the diversity of associated techniques (e.g., filter trapping, detergent treatments, centrifugation) make comparisons between studies difficult.

An assay developed by Alex Osmand, Johnson suggested, could offer a window into huntingtin's intermediate aggregate states. Osmand uses biotinylated poly-glutamine peptides at physiological concentrations to detect aggregation foci—sites that can actively recruit monomeric poly-glutamine molecules. Cuervo added that artificially blocking small oligomers from growing into large aggregates—by overexpressing chaperones, for example—might provide another means to study intermediate forms. Rubinsztein, however, considered that overexpressing chaperones could lead to secondary changes, making the results difficult to interpret. He also emphasized the need for developing kinetic assays to monitor huntingtin's many species dynamically through time.

A role for CMA in huntingtin clearance?

One clearance mechanism that may be important for clearing early aggregates, N-terminal fragments, and/or full-length monomers is CMA. Joan Steffan presented a series of observations her group has made in support of this possibility. Using an exon 1 huntingtin construct made byAlex Kazantsev, Steffan reported last year that SUMOylation of mutant huntingtin exacerbates neurodegeneration. Noticing that huntingtin's first 17 amino acids contain a cytoplasmic retention signal, as described by Truant, Steffan wondered if SUMOylation, which can occur on lysines 6, 9, and 15, might be blocking this signal and allowing huntingtin to move into the nucleus where it is toxic. Furthermore, she hypothesized that serine phosphorylation might be regulating the process by controlling SUMOylation, as reported for ataxin 1.

To test this possibility, Steffan generated several GFP-huntingtin constructs with mutated serines to mimic phosphorylation or de-phosphorylation states. The results indicate that phosphorylation of serines 13 and 16 regulates SUMOylation and nuclear localization. In addition, Steffan noticed an unexplained upward shift in her constructs with concomitant clearance. An anti-acetyl lysine antibody revealed that the constructs were being acetylated. In sum, the data indicate that huntingtin exon 1 is phosphorylated, acetylated and degraded. The

process is ubiquitin-independent and occurs more rapidly with constructs bearing 25 glutamines than with those bearing 47 glutamines.

Steffan speculates that the clearance might be occurring through CMA, as recently reported by Cuervo and colleagues for alpha-synuclein. She suggested acetylation could be creating a CMA consensus motif, noting acetyl-lysine is structurally very similar to glutamine. Indeed, when Steffan mutated lysines 6, 9 and 15 to glutamines, she observed a low molecular weight smear at the bottom of her gels, particularly when she mutated lysine 9. In addition, Steffan noted that the histone deacetylase inhibitor beta-hydroxybutyrate, which is known to ameliorate the HD phenotype, upregulates CMA. The compound has been thought to mediate its beneficial effects through histone acetylation, but Steffan proposed it could be acting through increasing huntingtin acetylation and its corresponding clearance. In addition, Steffan noted that the C-terminal Hsp70 (heat shock protein 70)-interacting protein (CHIP) appears to facilitate huntingtin clearance and wondered whether it could be accelerating CMA. Steffan's CHIP results are surprising, however, because she observed a CHIP-induced decrease in ubiquitination of huntingtin, whereas a previous study reported the opposite.

Steffan's current model is that huntingtin N-terminal fragments enter the nucleus where they are acetylated—possibly in promyelocytic leukaemia (PML) bodies by CREB binding protein (CBP)—and then cleared by CMA and/or PML body-associated proteasomes. PML bodies have been described as "nuclear depots" in which numerous proteins are recruited and/or released in response to stimuli such as viral attack or other cellular stress. Additionally, PML bodies appear to be sites for degradation of some nuclear proteins. SUMOylation targets proteins to PML bodies. Steffan suggested that cells may handle huntingtin as they do viruses, which are often targeted to PML bodies in early infection.

The proposal is far from confirmed, but Steffan noted some observations that are consistent with it. For example, Taylor observed increased clearance of the androgen receptor, a polyglutamine protein, in response to CBP over-expression. Furthermore, Yamamoto's identification of lamp 2a in her siRNA screens is consistent with a potential involvement of CMA in huntingtin clearance.

However, important uncertainties still remain. Cuervo and her colleagues have searched extensively for CMA involvement in the clearance of wildtype huntingtin, but have been unable to detect it. Although this might be explained by a failure of the antibodies used to detect modified, particularly acetylated, forms of huntingtin, the results are somewhat discouraging. In addition, Cuervo predicted that Fred Dice, a CMA expert, would probably not consider the motif described by Steffan a *bona fide* CMA signal.

In addition, even if CMA is shown to play a role in huntingtin clearance, it is still uncertain how significant it will be compared to other clearance mechanisms. As pointed out by Rubinsztein, most wildtype proteins are degraded by proteasomes and those that become aggregate-prone when mutated are often cleared by macroautophagy. If huntingtin follows this general tendency, CMA may account for only a small fraction of its degradation. Alternatively, as suggested by Johnson, proteasomes and macroautophagy may be the major players for clearing huntingtin monomers and large aggregates, respectively, but CMA may play an important role in clearing early protofilaments and/or N-terminal polyglutamine fragments.

Additional links between huntingtin and autophagy?

In addition to the possibilities discussed above, several other ways in which HD may be linked to autophagy were discussed. Participants noted that the metabolism of HD patients is

unusual. HD patients tend to lose weight, are diaphoretic (cool to the touch), and become hypoglycemic in the late stages of disease. Rubinsztein pointed out that presymptomatic patients have low body mass indices (BMIs), yet a Cushing's-like phenotype with increased central adiposity has been described in an exon 1 model of HD, and signs of it have also been reported in human patients. DiFiglia added that both symptomatic and presymptomatic patients are advised to eat large amounts of food, in particular high-fat foods, because increased weight seems to slow disease progression. As noted by Wexler, it is still unclear whether patients' choreic movements result in an increased consumption of calories.

One possibility is that these alterations are the direct result of hypothalamic dysfunction. As noted by DiFiglia, orexin-producing cells in the hypothalamus are damaged at very early stages of disease. If a disruption of orexin signaling is solely responsible for these metabolic alterations, autophagy's role may not be significant, as noted by David Ron.

However, if the apparently inefficient metabolism of HD patients is related to mitochondrial dysfunction, perhaps uncoupling, then autophagy might be very relevant. Hypomitochondrial function leads to insulin resistance, and insulin signaling is a strong inhibitor of autophagy. DiFiglia and Johnson noted that many studies have reported mitochondrial dysfunction in HD, but its importance as a primary or early mediator of disease has been questioned. Nevertheless, as pointed out by DiFiglia, imaging studies have revealed early energy defects in the brain and, working with lymphocytes, McDonald has found a linear correlation between ATP levels and CAG repeats. Cuervo added that the glucose dysregulation observed in HD might reflect an alteration in insulin signaling which, in turn, could affect autophagy. Yamamoto noted she has observed insulin-induced, rather than insulin-inhibited, autophagy in HD.

Of particular interest, Kegel noted that the insulin-like growth factor I (IGF-1)/Akt pathway has been implicated in the induction of macroautophagy under some conditions, and has been found to be progressively altered in HD. The second messengers phosphatidylinositol 3,4,5-triphosphate (PIP_3) and phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2] regulate many cellular processes via the activation of Akt. Interestingly, Kegel has found that huntingtin binds to phosphoinositides and HIP-1 binds preferentially to PIP_3. She also noted that autophagic vesicles accumulate PIP_3.

Participants considered that, in addition to following up on Kegel's studies, examining patients' metabolic profiles in greater detail might be worthwhile. For example, Yamamoto wondered if people with lower metabolic rates have a delayed onset of disease. Sulzer added that assessing amino acid metabolism—which correlates with autophagic activity—might be particularly informative.

A multitude of factors affect clearance mechanisms in HD

A major difficulty in understanding the role of autophagy and other clearance mechanisms in HD, and a probable reason for some of the apparently conflicting data, is that there are multiple factors affecting these processes. One of the most critical ones, as noted by Cuervo, is timing. It is very likely that at different stages of HD, different clearance mechanisms are activated or inhibited. The mechanisms are likely to change with time due to the dynamic interplay of pathological alterations and compensatory mechanisms.

Indeed, as previously mentioned, Taylor and Baehrecke have found that when proteasomes are inhibited, autophagy seems to act as an alternative pathway for clearance. The status of one clearance system may affect the status of another in a time-dependent fashion. For

example, as noted by Kopito, when lactacystine is used to block the UPS, there is initially no change in S6 kinase phosphorylation which induces autophagy in response to starvation. After approximately 8 hours, however, S6 kinase phosphorylation gradually increases, presumably mediating the activation of autophagy. Conversely, noted Nixon, inhibition of cysteine proteases causes an increase in the ubiquitination of at least some proteins. As noted by Talloczy, understanding the differential contributions of the various clearance mechanisms is of key importance.

The normal process of ageing may also have important effects on clearance and HD. Indeed, Yamamoto found that clearance of aggregates, after turning off mutant huntingtin expression in her inducible mouse model, was more effective in younger than older mice. Ageing results in decreased autophagy and a loss in the ability to upregulate autophagy properly, noted Cuervo.

Diet can also affect clearance and HD progression. Rubinsztein noted N171 mice who were fed low caloric diets lived longer and had less aggregates than those fed normal diets, potentially because starvation enhances autophagy. As noted by Cuervo, however, only certain caloric restriction regimens enhance autophagy—reducing daily caloric intake by 40% is very effective, whereas feeding animals every other day is not. Whether and how the high caloric diets of HD patients affect autophagy is unknown.

Yet another important consideration, noted by Cuervo, is that differences in experimental conditions may lead to apparently contradictory results. For example, her group has found that oxidative stress activates CMA. Cuervo noted there are many examples of proteins with multiple clearance targeting signals which are used differentially depending on the host cell's conditions. Consequently, it will be important to standardize conditions as much as possible.

What are the best model systems to study clearance mechanisms in HD?

Model system selection was also discussed at the workshop. As noted by Wexler, HD is a multi-faceted disease, inducing not only motor alterations, but cognitive and psychiatric disturbances. Although much research has been directed at understanding the vulnerability of striatal medium spiny cells, new findings are implicating a rapidly growing number of additional cells. For example, cortical and hypothalamic neurons appear to incur damage early on in the disease process, noted DiFiglia, and even non-neuronal cells show some pathological alterations. Indeed, Sulzer noted his group has observed an increase in macroautophagic vacuoles in HD cultured fibroblasts and lymphoblasts. Although cultured fibroblasts are unstable, with controls developing autophagic-like vacuoles after several generations, lymphoblasts have provided stable, clear-cut results revealing an HD-associated increase in macroautophagic vacuoles together with an approximately five-fold increase in protein degradation.When the cells are under stress, the results are even more dramatic. Sulzer is now interested in examining the effects of CAG repeat length and age.

These observations suggest that cell autonomous mechanisms, acting on a wide variety of cells, are key to HD pathology. Thus, it may not be necessary to limit model systems to striatal, or even neuronal, cells. On the other hand, as pointed out by Johnson, there is increasing evidence of neuronal circuitry playing an important role in HD. Thus, specific brain regions and their interactions may be critical to include when modeling the disease. Johnson noted that William Yang and his colleagues used the Cre/LoxP system to generate mice that express mutant huntingtin under the control of the nestin promoter (pan-neuronal model), or the Bmx promoter (cortical model). Phenotypic analyses of these models revealed aggregates, dysmorphic neurites,

shrinkage and dark cell degeneration in the cortical neurons of the pan neuronal model, but only aggregates in the cortical model. The implication is that pathological cell-cell interactions are critical to pathogenesis in HD. Several particpants noted, however, that the interpretation of these experiments is complicated. For example, Kegel pointed out that the nestin promoter is turned on for much longer than the Bmx promoter during development. It is thus possible that cells in the pan neuronal model are more damaged because they are exposed to huntingtin longer than cells in the cortical model.

As noted by DiFiglia, the existence of cellular mechanisms of pathology affecting single cells does not negate the importance of circuitry but, for the purposes of the meeting, she urged participants to focus on how to select models for studying the specific cell-autonomous process of protein clearance. Participants agreed that the key was to strike a balance between approachability and HD specificity. Kopito opined that much depends on the specific experimental question being asked. If axonal transport is being analyzed, neuronal cells will be necessary; if studying a more universal process, such as clearance, however, less limitations on cell type are required. Kopito stressed that, although neuroscientists have a tendency to focus on neurons' unique traits, neurons are, in many ways, very similar to other cells.

Wexler noted that non-neuronal cells from Venezuelan patients are available for future use, including cell lines from pre-symptomatics. In addition, Sulzer reiterated the fruitful experiments his group has performed using lymphoblasts, as well as these cells' superior stability compared to fibroblasts. (Extending these remarks, Ron and Kegel cautioned participants about cell lines undergoing chromosomal rearrangements and changing their phenotypes with passage number.)

The benefits of non-neuronal cells notwithstanding, several participants argued in favor of using neurons, even specific neuronal types, when possible. Steven Finkbeiner reminded participants that neurons are highly distributed cells, with synapse-associated functions occuring locally at great distances from the nucleus and affecting other cells. Sulzer agreed, noting that membrane trafficking in neurons is unique and of potentially great relevance to clearance mechanisms. Also, neurons do not respond to serum-deprivation by increasing autophagy as other cells do.

Based on his experience, Truant recommended a striatal-derived cell line created by Elena Cattaneo from an HD knock-in mouse generated by Marcy MacDonald. He noted that the cells are adherent, have long processes, good receptor expression, and show energy deficits and mitochondrial movement alterations known to be associated with HD. Jamshid Arjomand added that High Q is funding efforts to generate striatal- and cortical-like cell lines derived from human and mouse stem cells. When these cells are ready, they will be made available to the research community.

Action items

1. Extend microscopic observations of huntingtin-induced autophagy.
    a) Obtain quantitative microscopic data of autophagic structures to gain insights into the relative abundances of each, and their differential recruitment over time (Cuervo).
    b) Isolate autophagosomes. (Yamamoto)
    c) Examine Cuervo's suggestion that the smaller beaded structures observed by DiFiglia are associated with microautophagy by using appropriate markers at the

EM level. Obtaining a definitive answer, however, could be difficult. As noted by Cuervo, there is currently no good way to distinguish CMA from microautophagy *in vivo*.

   d) Examine the effects of CAG repeat length and age on autophagy in HD lymphoblasts (Sulzer).

2. Investigate in greater detail how mutant huntingtin affects autophagy.
   a) Assess how the degradation of various autophagy substrates, besides huntingtin, is affected by mutant huntingtin expression.
   b) Test mutant huntingtin's effects on fusion and trafficking. As discussed above, there are indirect observations relevant to this issue, but more direct experiments are needed (Sulzer).
3. Use mice crosses to probe autophagy's role in HD *in vivo*. Johnson suggested crossing autophagy mutants with HD mice. Yamamoto noted she is setting up to cross HD mice with mice that overexpress beclin-1. However, DiFiglia noted that beclin-1 is not essential for autophagy and Rubinsztein was concerned about potentially confounding side-effects (beclin-1 has been implicated in apoptosis).
4. Apply more sophisticated methods to determine the fates of full-length huntingtin and its fragments, in both wildtype and mutant proteins. (Sulzer)
5. Confirm results indicating autophagy is maximally induced in the presence of mutant huntingtin. To circumvent the caveat that one of the proteins being measured in this experiment is mutant huntingtin itself, Ron proposed shutting off huntingtin synthesis when adding the tracking label.
6. Extend studies of wildtype huntingtin's role in autophagy.
   a) Investigate how knocking down huntingtin affects autophagy.
   b) Determine how the amphipathic alpha helix identified by Truant is affected by polyglutamine expansion (Kegel).
7. Investigate the fates of intermediate oligomeric aggregates.
   a) Use Osmand's technique to examine aggregation foci (Johnson).
   b) Overexpress chaperones to block small oligomers from growing into large aggregates (Cuervo)
   c) Develop kinetic assays to follow the fates of different huntingtin forms over time (Rubinsztein)
8. Follow up Steffan's observations suggesting acetylation involved in huntingtin clearance by CMA. Steffan will collaborate with Cuervo to test CMA clearance of her mutant huntingtin constructs *in vitro*.
9. Investigate HD patients' metabolic profiles in greater detail.
   a) Assess whether patients with lower metabolic rates have a delayed onset of disease (Yamamoto)
   b) Evaluate amino acid metabolism (Sulzer)
10. Agree on the usage of terms to describe autophagy and standardize experimental conditions. Given that the study of autophagy is still in its infancy, there is a need to establish common guidelines and terminology to facilitate data comparisons and discussions between labs.

Therapeutic directions

Challenges

One of the major difficulties in designing ways to modulate protein clearance to treat HD is the lack of knowledge about the basic biology of different clearance mechanisms. Several participants pointed to our ignorance of autophagy's regulatory pathways as particularly problematic. As noted by Johnson, much would be gained by knocking down various negative regulators of autophagy to confirm the benefits of inhibiting mTOR activity reported by Rubinsztein, and to obtain new targets for drug development. Yet, as noted by Rubinsztein and others, almost nothing is known about what occurs downstream of mTOR. Even in yeast, one of the best understood systems for studying autophagy, there are many unresolved questions, such as the link between TOR and LC3, noted Kopito. The upstream regulation of TOR is also poorly understood. Baehrecke noted it is complicated and various factors impinge on its regulation.

In addition, as previously discussed, much remains unknown about the range of clearance mechanisms and their interactions. For example, Cuervo noted that new findings indicate there is cross-talk between autophagy pathways that hadn't been appreciated earlier. In particular, different spliced forms of lamp 2 are involved in regulating macroautophagy versus CMA. Thus, results obtained from lamp2 knockout organisms are messy and difficult to interpret. Also, very little is known about how different forms of autophagy and the UPS complement each other under different conditions. Nixon described the current understanding of the cell biology of autophagy as simplistic and noted there is a lack of knowledge of the diversity of autophagic mechanisms, a lack of markers to follow them, and a lack of consensus on the identity of different players.

Participants also discussed uncertainties associated with the reversibility of HD pathology. Although inducible mouse models have been very helpful, it is still unclear how early clearance mechanisms will have to be activated to effectively reverse, stop, or at least slow down disease progression. As noted by Rubinsztein, it is possible that after reaching a certain point in the disease, the damage caused by mutant huntingtin is irreversible and progression is inevitable. Thus, Rubinsztein and Reits considered it will be important to include presymptomatic patients in future clinical trials.

Another problem is that, in addition to its restorative function, autophagy can act as a mediator of cell death. As explained by Baehrecke, the two types of autophagy play important roles in development when massive amounts of remodeling occur. However, the differences between them are not well understood. Autophagic cell death is associated with caspase activation, but it is not simply the sum of survival autophagy and caspase activation. As mentioned previously, knocking out caspases still results in autophagy and cell death. This concerned participants who, like Yamamoto, worried about the possibility of inducing cell death in an attempt to bolster the clearance of toxic huntingtin species.

Even if inducing cell death can be avoided, enhancing survival autophagy may cause deleterious side-effects and fail to address primary toxicity problems, noted LeMaster. For example, a recent study by Teckman and colleagues suggests that, in α_1-antitrypsin (α_1-AT) deficiency, autophagy is induced by endoplasmic reticulum retention of α_1-ATZ which affects mitochondria, resulting in mitochondrial autophagy and injury. One possibility is that mitochondria are recognized nonspecifically by the autophagic response, which is constitutively activated to remove and degrade areas of the ER.

Rubinsztein tempered these concerns, and those regarding the potential induction of cell death, by noting that rapamycin, which crosses the blood-brain barrier, has been used for treating transplant patients for decades and no neurodegenerative side-effects have been reported. Nixon

suggested testing whether inhibiting autophagy exacerbates the disease as predicted. Rubinsztein has done this and found that overexpressing the small G protein rheb, which greatly enhances mTOR signaling, increased aggregate formation and cell death.

Another concern was brought up by Finkbeiner who asked whether tampering with autophagy might affect neuronal plasticity. Baehrecke pointed out that, although autophagy occurs during synaptic pruning in flies, it doesn't seem to be required, as indicated by genetic ablation experiments. Instead, glia dispose of synapses using standard phagocytosis. Nevertheless, autophagy may play other roles in plasticity. Rubinsztein noted that some *in vitro* studies suggest mTOR is important in LTP, and Sulzer noted that autophagic vacuoles are observed very distally in synaptic regions.

Treatment candidates

Rapamycin

As previously noted, Rubinsztein reported that rapamycin enhances the clearance of mutant huntingtin, protects against neurodegeneration in a fly model of HD, and improves behavioral symptoms in the Ross-Borchelt N171 mouse. Providing more of the study's details, Rubinsztein noted that mice were treated with CC1-779, an ester of rapamycin produced by Wyeth, beginning at 4 weeks of age and their performances on the rotarod test, grip strength test, wire maneuver test, and tremors were monitored up to 24 weeks of age. (CC1-779 is more stable and soluble in aqueous solutions than rapamycin and induces only mild side effects in humans. It is currently in clinical trials for cancer treatment.)

Johnson noted it will be helpful to know how rapamycin's effects compare to those of other therapeutic candidates, such as co-enzyme Q_{10}. Rubinsztein agreed and said he would investigate this further. However, he noted that assessing and comparing CC1-779's effects on survival, in particular, won't be easy because the United Kingdom requires researchers to euthanize mice before they reach late stages of disease. In addition, he noted that the drug treatments had to be limited because of weight loss. In fact, half-way through the trial, the researchers had to switch drug administration from three times a week to three times every other week to curtail weight loss. Even in control mice, rapamycin causes weight loss, perhaps by inducing cell shrinkage.

Rubinsztein said they are planning to refine the dosage to minimize this problem. One possible strategy, he noted, is to lower rapamycin dosage while combining it with other drugs that act through mTOR-independent pathways (see Inositol monophosphate inhibitors below). Participants also suggested ideas to investigate rapamycin's availability in the brain and optimize its dosage. Johnson proposed performing pharmacokinetic studies to determine the concentration of the drug in the brain. Rubinsztein said they have measured it six hours after injection, but have not studied the kinetics of elimination yet. He also noted that rapamycin has been shown to reach effective concentrations for the treatment of cancer in human brains, and that ongoing safety trials are being performed to ramp up the dosage as much as possible. Nixon proposed measuring autophagy in brain tissues from these subjects. To reduce rapamycin's rate of metabolism, Johnson suggested putting the drug in the animals' food and water, instead of injecting it.

Participants also discussed the use of additional mouse models to confirm Rubinsztein's results. Rubinsztein noted his team is interested in testing a full-length model of HD and asked participants for suggestions. DiFiglia recommended using a transgenic model with a robust motor phenotype and a knock-in model that develops the disease more slowly. In particular, she noted that the Zeitlin model has the human poly-proline region of huntingtin, which some

researchers believe is important, and displays early motor phenotypes that could be helpful for tracking disease severity. However, Johnson noted there is no clear evidence supporting the importance of the poly-proline region and added that the mice's deficiencies performing an open-field task are not robust. Moreover, the model's early rearing behavior is not well-suited for testing candidate treatments because it is not progressive.

Yamamoto pointed out that using a mouse model with a late onset may be desirable because age may be a key factor determining how the brain responds to HD. Rubinsztein agreed but noted that he needs a robust phenotype to effectively detect rapamycin's effects. Knock-in models or Hayden's YAC models, he fears, will not provide him with enough statistical power. Rubinsztein has examined the neuron counts and striatal volume effects in a paper published by Hayden, and come to the conclusion that he would need an unrealistic number of mice to obtain solid results.

R6/2 mice, on the other hand, have very robust motor phenotypes, but have other limitations. Rubinsztein's team decided against using this model because symptoms start very early, such that doing pre-symptomatic treatments would be difficult. In addition, R6/2's multiple peripheral symptoms could complicate the interpretation of the results. Johnson noted that a new version of the R6/2 model that develops the disease more slowly might be worth considering. He also noted that William Yang has a BAC transgenic mouse with a solid motor phenotype. However, the model has other limitations due to its high levels of huntingtin overexpression. Taylor suggested using a model from another polyglutamine disease, but participants were concerned that differences in mechanisms of disease may confound the results.

Participants also suggested examining whether other, non-motor biomarkers of disease might improve the power of late-onset, full-length models of disease. Yamamoto suggested monitoring electrophysiological changes and Nixon wondered if a more exquisite battery of behavioral assays, including cognitive tests, might provide increased sensitivity.

Also, DiFiglia noted there is a new inducible, full-length model of HD that may be worth investigating. Johnson added that such a model could be used to test whether rapamycin can speed recovery. Yamamoto said her group will be working with this model to repeat and extend their genetic screens.

Looking beyond these technical considerations, participants discussed rapamycin's general therapeutic potential. Participants agreed that Rubinsztein's experiments provide an important proof-of-concept for regulating clearance mechanisms to treat HD. In addition, rapamycin has the advantage of being a well-known drug that is already used chronically in human patients—its most widespread use is as an immunosuppresant for kidney transplant patients.

However, as noted by Johnson, an independent confirmation of the effectiveness of upregulating autophagy in HD, such as a demonstration that HD pathology is correspondingly altered when HD mice are crossed with autophagy mutants, would be helpful. In addition, Kopito noted that targeting regulators of autophagy that are downstream of mTOR would be better. Rubinsztein agreed, noting rapamycin is probably not the safest drug for long-term use as an inducer of autophagy. In addition to its immunosuppressant activity, it has various other side-effects. Participants also pointed out that mutant huntingtin itself inhibits mTOR activity to such an extent that starvation has little if any additional effects on autophagy. Thus, as noted by LeMaster and Sulzer, trying to inhibit mTOR further with rapamycin may not be the most effective therapeutic approach.

Inositol monophosphate inhibitors

Addressing this problem, Rubinsztein described an alternative, mTOR-independent pathway that also regulates autophagy. He noted that lithium, acting through the inhibition of inositol monophosphate which results in free inositol depletion and a consequent decrease in IP_3 levels, induces autophagy and enhances the clearance of mutant huntingtin. The involvement of this pathway is supported by experiments showing that an inostiol monophosphatase inhibitor, L-690,330, mediates similar effects. In addition, clearance was abrogated with either myo-inositol or prolyl endopeptidase inhibitor 2 (PEI), both of which result in the elevation of intracellular IP_3.

Baehrecke cautioned, however, that regulation of TOR can occur through various pathways that include complicated feedback loops. Thus, it is difficult to know with certainty whether lithium's effects are independent of TOR. Rubinsztein countered that they observe no changes in a TOR signaling component when treating with lithium. In addition, they observe lithium-induced autophagy even under conditions in which TOR is hyper-activated or hyper-inhibited. Still, Baehrecke was concerned about potentially unknown direct or indirect effects of lithium on TOR.

A few previous reports have studied the effects of mood-stabilizing medications on HD. Johnson mentioned a couple of studies using lithium in R6/2 mice. However, one report concluded lithium was deleterious, while the other claimed it was beneficial. Both studies' effects were very small. In addition, Steffan cited a single-case report published in the New England Journal of Medicine in 1976 indicating that valproic acid is beneficial for HD.

Participants suggested examining HD patient populations who have been on lithium for mood disorders. Rubinsztein said he is particularly interested in looking at patients treated with lithium before the onset of motor symptoms. Johnson agreed this would be of interest, but cautioned that the analysis would probably require a very large number of patients because it would involve predicting ages of onset to assess if they are delayed. Nixon added that it might also be useful analyze patients taking other mood-stabilizing drugs, such as carbamazepine and valproate.

Lipoic acid

Nixon asked whether lipoic acid, which has been reported to stimulate autophagy by Bergamini and colleagues, might be worth testing. Cuervo considered it worthwhile and added that Bergamini should be asked whether he has tested lipoic acid's effects on the brain.

Beta-hydroxybutyrate, ketogenic diet, and exercise

Based on her findings, Steffan proposed beta-hydroxybutyrate as a candidate drug to upregulate mutant huntingtin clearance through CMA. She also suggested a low-carbohydrate, ketogenic diet which increases beta-hydroxybutyrate levels. In addition, exercise might be beneficial because of its ability to boost reactive oxygen species (ROS) levels which, in turn, increase CMA.

Searching for additional therapeutic candidates

Participants agreed that seraching for additional therapeutic candidates is a priority. Johnson considered there is currently sufficient mechanistic understanding to conclude that the

general approach of upregulating clearance is worth pursuing. Now the focus, he stressed, should be on identifying specific, druggable targets and proving that clearance can be effectively upregulated in the brain.

Kopito considered that a large-scale consortium should be set up to perform small molecule discovery screens. Individual labs could contribute a variety of primary and secondary assays. Kopito noted his group, for example, has several fluorescent assays set up. In addition, Finkbeiner has developed an automated imaging and analysis system that enables the quantitative monitoring of individual cell fates and intracellular proteins over long intervals in a high throughput manner. Finkbeiner was interested in contributing to future drug screens, but noted that automating the analysis of some autophagy markers is difficult because their distribution changes from diffuse to punctate. Kopito pointed out that simply monitoring aggregate clearance using a GFP tag would be very useful. Finkbeiner agreed, noting that this approach would sidestep the uncertainties regarding the involvement of different clearance mechanisms. Once markers that can be reliably tracked are identified, monitoring huntingtin loss could be combined with tracking autophagy markers. Nixon added that using systems in which autophagy is down-regulated might be useful to enhance the screens' sensitivity.

Rubinsztein's team is already performing screens of various compounds, including small molecules. Together with candidate approaches, the screens have identified 12 drugs and 3 small molecules that cross the blood-brain barrier and induce autophagy. Rubinsztein predicts that some of these compounds are acting on targets that are safer and downstream from mTOR. In addition, Yamamoto noted that a screening effort to find HD candidate therapies involving 100,000 compounds and 10 centers across the country is already underway supported by the NIH's Molecular Libraries Screening Centers Network (MLSCN). Individual labs write grants to include their assays in the study. However, she alerted participants that as soon as a hit is identified, it is reported online.

Participants were positive about performing blind screens which, as noted by Kegel, often reveal unexpected drugs and targets. But they also pointed out some associated limitations. As noted by Johnson, blind screens result in lead compounds with unknown targets, which can be problematic. The pharmaceutical industry is mostly unwilling to invest in them because of the difficulty of finding their targets, which is necessary for optimizing their performance. However, Kopito stressed that, regardless of these considerations, compounds with robust effects should be searched for and developed because the alternative of ignoring them is not acceptable. Finkbeiner agreed, noting that many tools exist today to find targets, such as siRNA and yeast systems. Johnson acknowledged this point, but argued that a strong mechanistic foundation is required to pursue candidate drugs. Screens can be fairly loose, he noted, but follow-up studies should be limited to those compounds with a clear mechanism of action. In this context, Johnson noted that genetic screens are desirable because they provide targets upfront. He considered that using genetic screens to identify downstream negative regulators of autophagy will be particularly valuable.

It is also important to note that screens for regulators of other processes may yield useful candidates. For example, Reits pointed out that finding compounds that increase huntingtin monomerization might be of therapeutic value, as they could make mutant huntingtin more accessible to cleavage by TPPII.

Clinical trials

Although, as mentioned above, much remains to be done to design ways to regulate protein clearance as a therapeutic option for HD, Johnson noted it is not too early to begin thinking about clinical trials. Mice data are not required by the FDA, and Johnson considered there is currently a sufficiently well-supported mechanistic rationale to warrant clinical projects. To help guide efforts in this direction, Johnson summarized the drug development schedule that Novartis, who is working with the HDF, plans to follow. Initially, the company expects to conduct trials 2 to 4 weeks long to confirm that candidate compounds reach the brain and have the expected mechansitic effects on their targets. In the second stage, trials lasting 6 months to a year will test whether candidates prevent striatal shrinkage as assessed by imaging techniques. Only after completing these two stages successfully will full-blown trials be initiated.

Rubinsztein considered that the initial stage is tractable and, if necessary, a primate trial could be run to demonstrate a compound's mechanistic effects. However, he considered that it will be difficult to achieve enough power to successfully complete the second stage trial. Although Johnson noted that calculations indicate that 6 months to a year should be sufficient time to detect solid therapeutic effects, Rubinsztein worried that moderate, but significant, effects could be missed due to patient variability and a lack of biomarker sensitivity. As an alternative, he proposed using patients suffering from ataxin-3, a disease likely to share similar clearance problems with HD, but which has an ostensibly cleaner phenotype and a more clearly defined onset.

As pointed out by Kegel, biomarker quality is indeed a major problem in HD. Many detectable alterations are variable and do not track progression linearly. Recognizing the importance of this problem, the High Q Foundation is funding several projects to identify biochemical markers (using metabolomic, proteomic, and microarray data) in plasma and cerebrospinal fluid, as noted by Arjomand. Within a year, they expect to have useful results they will share with the research community.

In addition, imaging techniques are providing great hope for obtaining early biomarkers that track disease progression well, noted DiFiglia. Chris Ross and colleagues have found that striatal volumes begin to atrophy at least 10 years before the onset of motor symptoms and decline steadily during the presymptomatic stage. By the time of motor onset, striatal volumes are approximately half the normal size. Diana Rosas and colleagues have observed similar changes, in addition to cortical changes that may occur even earlier. Johnson said that within a year imaging is expected to become a key tool for tracking HD progression.

Participants speculated whether imaging could also be used to help track changes in metabolism associated with autophagy. Nixon proposed monitoring brain metabolism, in conjunction with striatal volume, and wondered if there are PET ligands that could serve as indicators of autophagy. Rubinsztein noted that analyte-binding dyes that have been used to study Alzheimer's disease might also be useful for HD.

Participants also discussed whether psychiatric alterations might provide early HD biomarkers. As noted by Nixon, neurons involved in mood regulation may be particularly vulnerable to stress and, thus may be affected in a wide variety of neurodegenerative diseases. Mood alterations are often discounted as reactive, he said, yet increasing evidence indicates they may be part of the disease process and even serve as valid indices of progression. Wexler pointed out that HD almost certainly has an important early psychiatric component, noting that the suicide rate of people carrying the HD mutation, but who have not expressed the disease, is much higher than that of siblings who do not carry the mutation. Yet mood alterations often go untreated in HD because physicians focus on HD's motor symptoms. DiFiglia added that the

basal ganglia receive serotonin inputs, and new studies indicate HD pathology associated with limbic-basal ganglia connections. Whether psychiatric disturbances can be used as biomarkers of HD, however, is unclear. Wexler noted that there is huge variability in the presentation and evaluation of these problems.

Action Items

1. Follow up on rapamycin studies
    a) Refine rapamycin dosage to minimize weight loss (Rubinsztein)
    b) Perform pharmacokinetic studies of rapamycin's uptake and clearance in the brain (Johnson)
    c) Administer rapamycin in food and water (Johnson)
    d) Evaluate autophagy levels in brain tissue from subjects treated with rapamycin (Nixon)
    e) Compare rapamycin's effects in mice to those of other therapeutic candidates, such as co-enzyme Q_{10} (Johnson)

2. Extend findings using inositol monophosphate

Examine disease onset and progression in patients treated with lithium before the onset of motor symptoms (Rubinsztein).

3. Search for more and better downstream drug targets
    a) Set up small molecule discovery screens (Kopito). Consider recruiting Finkbeiner's imaging and analysis system and Kopito's fluorescent assays.
    b) Set up genetic screens (Johnson, Yamamoto)
    c) Continue analyzing data from Rubinsztein's candidate and blind screen approaches

4. Keep abreast of the development of biomarker candidates

In particular, the refinement of brain imaging techniques and High Q's searches for biochemical markers.