Workshop Report: Autophagy and the Huntingtin Life Cycle
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CHDI Los Angeles
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Executive Summary

On June 20-21, 2011, CHDI hosted a workshop in Los Angeles focused on autophagy. Cells use this process of "self-eating" to clear waste materials from the cytoplasm, including pathogens, dysfunctional organelles, and aggregate-prone proteins—like mutant huntingtin (mHtt). Participants included experts in the fields of autophagy, Huntington's disease (HD), and drug development. The group came to a somewhat surprising level of consensus that autophagy indeed represents a viable and potentially valuable target for therapeutics aimed at HD. To begin the workshop, Allan Tobin asked the fundamental question: why should CHDI be interested in autophagy as an HD target? David Rubinsztein answered that mHtt has been demonstrated as a good autophagy substrate, and degradation is relatively selective for mutant vs. wildtype huntingtin. Moreover, targeting autophagy might represent a multi-pronged strategy. Macroautophagy (including both non-selective "bulk" macroautophagy and selective macroautophagy), chaperone-mediated autophagy (CMA), and microautophagy comprise distinct autophagic processes, each of which may be targeted in different ways that might provide benefits in the HD brain. During the workshop, participants discussed what's known about these processes and how they might be targeted to reduce mHtt. Robert Pacifici also posed two

questions early in the workshop. First, can autophagy be stimulated, or is it already working at a maximal level? If it can be increased, would this provide beneficial effects for HD? And second, in the setting of HD, is autophagy fundamentally broken? The basic answers to these questions are that yes, it can be successfully and even safely upregulated with beneficial effects in the setting of mHtt aggregation. And yes, mHtt does cause fundamental, non-neuronal-specific defects in autophagy, but apparently only in selective macroautophagy.

Participants also identified weaknesses that need to be addressed in developing potential therapeutics for HD aimed at autophagy. By nature, autophagy is a dynamic process that must somehow be measured as such. Static snapshots of the process do not provide the same information that visualization or measurement of flux can provide. Until recently, no reliable assay had yet been available to measure autophagic flux, particularly in neurons or in living animals. Likewise, the synthesis, secretion, processing, aggregation, and degradation of the huntingtin protein (both wildtype and mutant) make up a dynamic process that is not yet understood. Assays to track Htt through its life cycle would aid in targeting the toxic protein species—which is not yet identified. In addition, ways to measure levels of Htt protein from living humans will be key to both developing biomarkers of HD and finding read-outs of autophagic activity associated with Htt.

Introduction

To begin the workshop, Robert Pacifici laid out three main "tiers" of the CHDI strategy. The first two tiers involve lowering levels of toxic mutant huntingtin, either directly or indirectly, as a therapeutic strategy. The third tier of research is focused on other processes and molecules—including potential targets—that are involved in HD pathology. CHDI has chosen to bank on three major processes: bioenergetics, synaptic dysfunction, and autophagy. Pacifici wants to tailor CHDI's research of autophagic processes related to HD; he presented participants with these key questions: can autophagy be stimulated from its operational level in HD, or will stimulation likely reach a ceiling effect? Secondly, assuming it can be increased, is it broken in HD? Would increased autophagy result in beneficial clearance of mutant Htt?

David Rubinsztein addressed Allan Tobin's fundamental question: why should CHDI be interested in autophagy as a target for HD therapy? Mutant huntingtin (mHtt) is a good autophagy substrate—including both fragments and full-length protein—and "the attraction is that it's relatively selective for mutant vs. wildtype huntingtin." "Autophagy" actually describes a diverse set of degradation pathways including nonselective "bulk" macroautophagy, selective macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy, each of which will be considered. Though autophagy processes vary in their target selectivity, Rubinsztein suggests that even non-selective macroautophagy could be selective for clearance of mutant

protein compared to wildtype. As Allan Tobin noted, the capacity of the autophagic pathway to clear protein aggregates may provide a unique strategy for selectively clearing both precipitated and soluble mHtt (if the two forms undergo metabolic exchange *in vivo*) without depleting the cell of wild type Htt. Workshop participants shared their expertise to describe how selectivity is achieved in each autophagic pathway, and how the process might be manipulated with respect to HD.

Ai Yamamoto described work that she published 11 years ago (Yamamoto et al., 2000). In an inducible mouse model of HD, when expression of Htt was halted, inclusion bodies disappeared and the disease phenotype was apparently reversed, suggesting that the disease process is at least somewhat reversible with protein clearance. Could stimulation of autophagy be a route to therapeutic clearance? Several labs' work consistently suggests that full-length huntingtin is clearly targeted by the autophagy pathway, as it is contained in autophagosomes. David Rubinsztein and others confirm that autophagy can easily be upregulated both chemically and genetically in neurons or in brain, but this has not yet been achieved in an HD setting.

Autophagy in HD: is it broken?

Many CHDI representatives had Pacifici's basic question in mind throughout the workshop: is autophagy "broken" in HD, or are there at least "bottle necks" that are preventing efficient clearance of mHtt? Ana Maria Cuervo spoke about work (Martinez-Vicente et al., 2010) in which she identified a cargo recognition defect in selective macroautophagy in many HD model systems, including mouse fibroblasts, liver cells, primary cultured neurons, striatal cell lines, and lymphoblasts from ten HD patients. She did not find deficits in autophagosome formation, in autophagosome fusion with the lysosome, or in pH of the lysosome itself. The deficit in these models appears to be limited to cargo recognition. "It seems with altered Htt, the polyQ is messing up the ability to recognize organelles," she said. Recognition of aggregated, poly-ubiquitinated protein was also impaired. Importantly, mutant Htt causes this defective selective macroautophagy in peripheral, dividing, non-neuronal cells as well as neurons. So while the autophagic defect in HD appears to be confined to a specific mechanism, it could have far-reaching consequences. Accumulation of dysfunctional mitochondria and other organelles could account for the disrupted cellular bioenergetics or oxidative stress seen in HD. Cuervo is pursuing the nature of the mHtt interaction that leads to disrupted cargo recognition. The experiments were conducted using expression of full-length huntingtin with polyglutamine expansion lengths of Q18 or Q7 and Q111. Cuervo reports that, in these systems, nonselective "bulk" macroautophagy appeared functionally intact (because soluble cytosolic proteins appeared normally in autophagosomes), as did chaperone-mediated autophagy (CMA).

Nonselective macroautophagy

Many distinct forms of autophagy have now been characterized. The originally described process called autophagy we can now differentiate as non-selective or "bulk" macroautophagy. The basic steps to macroautophagy include formation of an autophagosome, uptake of bulk cytoplasm containing misfolded or otherwise dysregulated proteins, transport of those contents to the lysosome, fusion with the lysosome, and subsequent degradation of the materials by low pH and proteases contained within the lysosome. Canonical autophagy genes (Atg) and the proteins they encode have been characterized in many systems. The genes involved in the signaling and transport complexes appear to be conserved between yeast and mammals.

Yamamoto's 2000 work suggested that this basic autophagic process works to clear Htt. In that study, when expression of mHtt was halted in cells, aggregates were completely cleared within a few days in HeLa cells and in six days in neurons. Other data also suggests that non-selective autophagy likely works to clear polyQ proteins. A GFP-tagged polyQ tract, which contains no lysines and therefore is not ubiquitinated, is still cleared in an autophagy-dependent fashion. Rubinsztein suggested at the outset that even this bulk autophagy mechanism has some intrinsic selectivity for mutant Htt. He has seen, in multiple systems, that stimulation of non-selective macroautophagy appears to clear mutant Htt with no effect on clearance of wildtype Htt. How this selectivity for mutant over wildtype Htt arises remains unclear, but one possibility is that macroautophagy preferentially clears soluble rather than aggregated proteins. Cuervo outlined a scenario in which mutant huntingtin protein is in some dynamic exchange in and out of the aggresome, and its particular form may determine its degradation by specific autophagic pathways. Even this nonselective process may thereby "target" a population that contains more mutant than wildtype Htt. Hellerstein reminded the group that the degree of dynamic exchange between aggregated mHtt and monomeric or multimeric forms of mHtt has not yet been determined, although the tools are now available for doing so (see below). Yamamoto and Rubinsztein agreed that macroautophagy likely clears soluble monomeric, multimeric, and aggregated subspecies of proteins interchangeably.

Does the size of the aggregates matter in terms of clearance? Ana Maria Cuervo suggests that aggregate size is less important than the mobility of its protein contents. Tamotsu Yoshimori reported that he has never seen large aggregates surrounded by a large autophagosome. One possibility is that the autophagosome removes smaller pieces of the aggregate rather than engulfing the whole structure. But Andrey Tsvetkov reports that he has seen neuronal inclusion bodies appear within 24 hours and these huge structures completely disappear suddenly; they don't get smaller slowly. The cell may use a strategy to somehow put its trash into a trashcan and get rid of it all at once. In Yamamoto's work showing disappearance of inclusion bodies after halting mHtt expression in HeLa cells, they never visualized aggregates larger than 1 μm (by electron microscopy, EM). She pointed out that

there is likely a biophysical size exclusion issue. One speculative mechanism for why selective autophagy (see below) requires molecules like p62 and Alfy is that they provide a structural scaffold for the formation of vesicles that can in turn be engulfed by the autophagosome.

A distinction should be made between basal and induced autophagy. Most studies of autophagy have examined the process after a manipulation to activate the pathway, including various cellular stresses, starvation, or inhibition of the mammalian target of rapamycin (mTOR), a negative regulator of autophagy. While these have yielded valuable information, says Zhenyu Yue, it may be as valuable in the HD setting to understand autophagy in its homeostatic, basal setting, particularly in neurons. To underscore that point, several participants raised the point that autophagy—or at least regulation of nonselective macroautophagy—appears to differ in neurons compared to non-neuronal cells. Ivan Galimberti highlighted the fact that although much is known about autophagy in non-neuronal cells, we are just beginning to describe how autophagy is regulated in neurons. Specifically, while starvation readily stimulate autophagic clearance in most cells, starvation is not a potent activator of autophagy in the brain. This could arise from neurons' extensive protection from nutrient deficiency, so that starvation is not a normal trigger of cellular autophagy in the brain. Likewise, the potent activator rapamycin only weakly activates autophagy in neurons. And significantly, the canonical marker of autophagy used in peripheral cells has been microtubule-associated protein 1 light chain 3 (LC3), a component of the autophagic complex that follows the pathway from induction to degradation in the lysosome. Lipidation of LC3, or conversion of LC3-I to LC3-II, cannot be reliably triggered or visualized in neurons, even with the phosphorylation and dephosphorylation events regulated by mTOR that "should" trigger conversion. "Some scientific knowledge is missing," in our understanding of autophagy in neurons, Galimberti added, "and additional studies will reveal how neuronal flux responds to a variety of potential inducers."

Though much information has been gathered to describe the process of autophagy, we still lack a cohesive understanding of the dynamic processes. Tibor Vellai has undertaken efforts to model the autophagic pathway. Several participants described agents known to influence autophagic processes. Those that affect nonselective macroautophagy are discussed here. Rapamycin is perhaps the best-known molecule to affect autophagy. It acts as an inhibitor at the mammalian target of rapamycin (mTOR), a serine/threonine kinase that plays a key role as a negative regulator of bulk macroautophagy. It regulates the phosphorylation of key autophagy pathway components including Atg1, ULK1, and probably ULK2. In addition to autophagy, mTOR is involved in regulation of other cellular processes including cell growth, survival, and protein synthesis. mTOR acts as a sensor of nutrient levels, and is the control point for autophagy induced by either starvation or rapamycin (at least in non-neuronal cells). Rapamycin has been used extensively

as an FDA-approved drug for immuno-suppression during organ transplant, and has been shown to extend lifespan in animals. However, very high doses are required in people to reach the CNS, and it remains unclear whether rapamycin activates autophagy in neurons in the same way that it does in non-neuronal cells. The question then remains, is mTOR a good target for autophagy in HD? Rubinsztein described mTOR as "pretty close to the autophagy pathway," but Vellai and others felt that mTOR was not an optimal target for regulation of autophagy in the brain because of its upstream position and its effects on other cell processes. Rubinsztein screened small molecules for substrate clearance and identified the hypertensive drug rilmenidine as an autophagy stimulator. Its target is a G_i-coupled protein receptor. He reports that clinical trials will begin shortly.

Autophagy, and formation of the autophagosome in particular, can be controlled by levels of phosphatidylinositol 3-phosphate (PI3P). Both kinases and phosphatases could be manipulated to influence autophagy induction. Tamotsu Yoshimori explained, "We think there's a balance of PI3 kinase and phosphatases [activity] that's important to form autophagosomes, because PIP3 is a component of the autophagic complex." The relevant phosphatases include a group called myotubularin-related genes. Yoshimori has identified Jumpy and other phosphatases as negative regulators of autophagy. Vellai has also identified candidates from this class of phosphatases including MTM4 and MTM8. He described a future aim to block the phosphatases with small proteins or perhaps specific small molecule compounds to regulate the molecules as a way to stimulate macroautophagy.

Yoshimori identified another possible regulatory point with his characterization of Rubicon. The protein negatively regulates autophagy, and this function depends on binding to ubiquitin. Yoshimori has now identified a small domain required for this binding. He envisions that a small peptide created to bind this region could prevent Rubicon binding and thereby release inhibition of autophagy. Rubicon appears to regulate fusion between the autophagosome and endosomes and also between autophagosome and lysosomes, acting as a scaffold.

Selective macroautophagy

Selective macroautophagy differs from nonselective macroautophagy in that it requires the specific recognition of cargo. Autophagy receptors interact directly with cargo and with LC3 via a specific LC3-interacting region typical for all autophagy receptors. Yoshimori outlined three types of selective autophagy: xenophagy aimed at pathogens including bacteria; mitophagy aimed at dysfunctional mitochondria; and aggrephagy, selective for aggregate-prone proteins. Other selective autophagic processes target various intracellular materials for degradation. Anne Simonsen and Ai Yamamoto have found that selective degradation of aggregate-prone proteins depends on Alfy (autophagy-linked FYVE protein), a phosphatidylinositol 3-phosphate (PI3P)-binding protein. Simonsen

believes that Alfy may regulate the level of selective vs. nonselective autophagy in the cell, because Alfy is normally "hiding" in the nucleus, and is then recruited out to the cytoplasm during certain cellular stresses, including accumulation of protein aggregates. Although complete loss of Alfy is perinatal lethal, Alfy does not modulate the non-selective starvation-mediated autophagic response and is not part of the core autophagy machinery itself. Alfy competes for binding with the core autophagic machinery, so sequestration from the cytoplasm may allow the cell to mount a proper response to other non-selective stresses like starvation. A nuclear localization sequence (NLS), a nuclear export sequence (NES), and post-translational modifications of Alfy all present potential manipulation sites that might be exploited to increase specific clearance of mHtt.

While Alfy may or may not be an autophagy receptor itself, it interacts with key components of well-characterized autophagy machinery. Importantly, it also interacts with mutant huntingtin aggregates. Simonson explained, "We believe Alfy can bridge accumulating Htt proteins to the autophagic membrane and machinery by forming a scaffold complex including p62, LC3, Atg5, and PIP3." With depletion of Alfy, degradation of mutant Htt (and other aggregate-prone proteins) is reduced, resulting in increased aggregates. With overexpression of Alfy, clearance of aggregates is increased in HeLa cells, in primary neurons, and in the *Drosophila* eye model of HD. Importantly, soluble levels of Htt exon-1 25Q were not affected by Alfy depletion, suggesting that Alfy does not target soluble protein for clearance. Alfy is a large protein with functional domains found only in the C-terminus, and an N-terminus interaction with aggregated Htt. The nature and specificity of this interaction remain unclear. Regulation of export of Alfy from the nucleus is not yet understood, but the autophagy protein p62 is required for export; Alfy and p62 appear to be constitutively bound. Alfy appears to enhance target recognition in selective autophagy: over-expression of the C-terminal region results in increased clearance of polyQ proteins. Removal of the autophagy protein Atg5 from the system eliminates this effect, suggesting it is macroautophagy-dependent.

Ai Yamamoto described the work they've done to relate their results in HeLa cells and cultures to the adult brain using a genetic approach to manipulate Alfy levels. The goal is to understand how modulating Alfy will modulate HD phenotype. Overexpression of Alfy led to improved aggregate clearance, so would increased Alfy levels also lead to improved phenotype? She has begun to examine this question in both the fragment and full-length BAC-HD models. Another question she has addressed is, does elimination of Alfy worsen phenotype progression in a mouse model of HD? In mice with 50% knock-down of Alfy crossed onto the full-length BAC-HD model, the rate of onset of phenotype was significantly increased, in measures including increased weight gain, feeding behaviors, and movement phenotypes. The question of whether protein aggregation is accelerated will be determined when the mice are sacrificed at the age of 11-12 months, after their behavioral studies are completed.

Alfy is constitutively expressed and normally confined to the nucleus, where it is found co-localized with p62 at PML nuclear bodies (also called nuclear dots or inclusions) that contain misfolded proteins. One possibility is that Alfy and p62 might be required to shuttle nuclear proteins to the cytoplasm for degradation. Dimitri Krainc also mentioned work with Francis Collins in which they showed that the nuclear protein progerin (the mutant form of lamin A) was ubiquitinated, bound to p62, and transported out of the nucleus. In the cytoplasm, Alfy co-localized with progerin-positive structures, suggesting a possible role for Alfy in translocation of progerin. A question remains regarding the role of Alfy-mediated selective autophagy aimed at aggregate-prone proteins in HD. This would seem to be a major clearance pathway, so why isn't it clearing mHtt efficiently? Is the polyQ expansion interfering with clearance of mHtt and other substrates (perhaps preventing cargo recognition), or is selective autophagy simply overwhelmed by excess aggregated protein? Cuervo's work seems to suggest that cargo recognition of both aggregated proteins and organelles may be compromised by mHtt.

Tamotsu Yoshimori has focused his efforts on autophagy of pathogens, but this bacterial clearance may reveal common systemic targets or ways to upregulate selective autophagy. Yoshimori also recounted an experiment in which he injected soluble recombinant polyQ to brains and found that aggregates formed within 24 hours, but before that, the injection itself caused an increase in macroautophagy, leading him to believe that micro-aggregates of polyQ were targeted by selective autophagy. Ana Maria Cuervo suggested that perhaps dynamically mobile proteins leaving large aggregates are specifically targeted by autophagosomes. Mauro Piacentini suggested that specificity of the process could be linked to ubiquitination, which provides a marker for species that should be eliminated. However, many substrates of macroautophagy don't require ubiquitination. Rubinsztein asked whether increased ubiquitination would enhance clearance. Gill Bates added that the data isn't clear when it comes to whether or not mutant Htt is ubiquitinated, or whether protein ubiquitination increases with polyQ expansion. Experiments that quantify ubiquitinated Htt, whether wildtype or mutant, are technically difficult to perform. Krainc believes that Htt is ubiquitinated at specific lysines, but the downstream effects are unknown. Nukina identified phosphorylation of p62 as a modulation point for selective autophagy. He suggested that identification of specific sites of phosphorylation of p62 might reveal ways to increase selective autophagy to degrade ubiquitinated proteins including mHtt.

Chaperone-mediated autophagy (CMA)

Chaperone-mediated autophagy (CMA) might also be a mechanism to improve clearance of mutant Htt. CMA differs significantly from macroautophagy in that protein unfolding is required for translocation into the lysosome. An Hsc70-containing chaperone protein aids in unfolding and presents its cargo to the only known lysosomal receptor, the lysosomal-membrane protein type 2a (LAMP-2a).

Ana Maria Cuervo and Nobuyuki Nukina have explored the clearance of Htt by CMA. While Htt contains four pentapeptide motifs (the recognition sequence for CMA), wildtype Htt is not targeted by CMA in the cell, because these motifs are cryptic. In HD models, CMA was found to be upregulated, probably as a downstream result of compensatory crosstalk with other autophagic mechanisms. If toxic protein fragments could be identified and targeted, CMA might provide a way to clear them.

Nobuyuki Nukina originally designed the polyQ-binding peptide (QBP) to block aggregate formation. QBP contains a recombinant expanded polyglutamine-specific binding site. He predicted that expanded polyQ would be degraded by CMA, but targeted degradation by CMA requires Hsc70, so they incorporated this motif into a QBP linker molecule. Expression of this QBP1 in R62 mice decreased mHtt aggregate formation and improved survival. The benefits might arise from both removing abnormal protein via CMA and from blocking the process of aggregation itself. However, this blockage may also be inherently dangerous if toxic intermediate species form (of QBP and polyQ proteins) and are not degraded. The aggregates must somehow be linked to a degradation system. This strategy would require a gene-therapy approach.

Microautophagy

Ana Maria Cuervo has also started looking at the process of microautophagy. Distinct from macroautophagy, microautophagy sequesters cytoplasmic materials directly into an existing lysosome or late endosome. Also unlike macroautophagy, evidence suggests that the molecular components of the pathway in yeast are not conserved in mammals. The budding-in process can take in bulk cytoplasm and can also capture materials selectively, which requires an Hsc70 motif for recognition, just as CMA does. The resulting vesicular bodies that form in the late endosome could be directed to a lysosome for complete degradation, or the endosomes could fuse with the plasma membrane and release the vesicular bodies outside the cell. The deposited vesicular matter may then be degraded by extracellular proteases or neighboring cells, perhaps including astrocytes or other glia. Current studies on microautophagy are focused on determining whether microautophagy can be upregulated as a result of compensatory crosstalk mechanisms, so a clearer picture of the native processes may emerge with further inquiry.

Crosstalk

Bob Hughes raised the question, if you measure for example a 10% reduction in mutant huntingtin due to increased autophagy, and you see a corresponding improvement, is that improvement attributable to the 10% reduction in the mutant protein itself, or is it due to an overall change in the cell's physiology, downstream of all its substrates? Participants agreed that improvement would likely arise from both sources, and Ana-Maria Cuervo pointed out that each pathway does not operate in isolation. If an overall effect on homeostasis produces an improvement by affecting various substrates and processes, it won't matter in the long run if they're

not all attributable to autophagy. Perhaps a more immediate question is, will manipulation of various forms of autophagy result in compensatory mechanisms that would erase any potential benefits? It seems that while there is extensive crosstalk between the various autophagic pathways, this problem would not arise from upregulation of autophagy. Block of autophagic pathways tends to cause compensatory increases in parallel pathways, but over-stimulation does not appear to cause concomitant decreases in other pathways. For example, Rubinsztein reports that with inhibition of autophagy, he sees "a tiny bit" of accumulated wildtype Htt, likely because block of autophagy leads to block of the ubiquitin proteasome system (UPS). At the same time, over-activation appears not to be a concern, as strict regulatory mechanisms exist. Participants agreed that experiments would lead most directly to a therapeutic strategy if they approximate the physiological situation, using expression of endogenous full-length Htt with a relevant CAG length.

Allan Tobin asked, in order to identify Htt as a substrate of a specific pathway, could one saturate the autophagosome system to "soak up the autophagic bandwidth" and observe whether Htt accumulates? The problem with this approach is that no substrates are strictly specific for one autophagy pathway without clearance by another pathway. Inhibition of the UPS is not an advisable strategy, because cells become sick before autophagy is activated. Yue believes that constitutively active, basal autophagy might degrade some proteins more readily than others, and several participants agree that it goes back to the size and mobility issue. Hellerstein suggests that with his tandem mass spec technique, he could identify the primary substrates that are influenced by autophagy by looking at protein turnover from beginning to end in a given cell or tissue population by using knockdown of autophagic components. However, Cuervo cautioned that degradation of substrates might not be affected by removing one autophagic pathway, because compensatory mechanisms would degrade them. Additionally, some genes are absolutely essential for autophagy, but they must be reduced by 95% to see the loss of autophagy.

What is the toxic species in HD?

While Yamamoto's work (2000) suggested the promise of reversibility of neuronal toxicity in HD, it did not reveal the nature of that cellular toxicity. Yamamoto explained, the phenotype reversal arose from clearance of every possible mHtt protein: new protein was not created, and existing protein was all cleared, including aggregated and soluble species. Ethan Signer spelled out this key unanswered question: what is the toxic species in HD? We don't know what that protein is, whether it's a fragment, if it's soluble, if it forms oligomers or aggregates, or whether the aggregates themselves are toxic. Clearly, levels of cellular huntingtin are difficult to measure and difficult to analyze, and at any give time might represent a heterogeneous population of peptides. While measuring the turnover of various Htt proteins may be helpful to an overall understanding, the key issue for HD is clearance of the toxic protein species. However, this lowered toxicity might be achieved directly or indirectly. Tobin pointed out that if protein species are in some

dynamic equilibrium between aggregates, oligomers, and monomers, it might not matter what the toxic species is. If you can reduce mutant Htt at any point in this dynamic exchange, you may reduce the level of the toxic protein species, whatever it may be. Thus, the efficacy of any huntingtin-lowering strategy might depend on how quickly the protein species equilibrate between soluble and included (aggregated) Htt. This sort of a systems approach highlights the importance of dynamic measures of protein flux within cells. (One suggestion for how this might be achieved would be to knock in a mutant Htt containing a fluorophore so that one could track soluble vs. aggregated protein in real time.)

Though questions remain, there is some evidence regarding the toxicity of various proteins. In efforts to reduce mHtt aggregates, Nukina has conducted experiments to separate the aggregates into its two components: insoluble and soluble. Nukina expressed his belief that the soluble component is more toxic, "but still, we don't know." Nukina also predicted that removing aggregates would reduce toxicity to the cell. The Finkbeiner group has shown that inclusion bodies are themselves neuroprotective, and that diffuse (soluble) mutant huntingtin is toxic. By the optical pulse-chase method (see below), Tsvetkov and Finkbeiner showed that protein half-life, rather than expression level, was more influential in neurodegeneration. Soluble, longer-lived mutant proteins proved more detrimental. (Paradoxically, soluble wildtype Htt, which is presumably non-toxic, has a longer half-life compared to mutants.) Bob Hughes agrees that blockage of axonal processes by aggregates is most certainly bad for neurons, but wonders if the situation might be made worse if toxic protein were mobilized out of inclusions.

While the toxic mutant Htt species remains unknown, toxic processes should also be considered. Rubinsztein and Nukina agreed that the aggregation process itself could contribute toxicity distinct from potentially toxic mHtt species. This again highlights the importance of measuring *flux* of dynamic autophagic and aggregation processes rather than absolute levels of soluble or accumulated proteins. The rate of aggregation may be more important than the final results of the process. For example, said Rubinsztein, one possibility is that mutation results in a short-lived intermediate species with a high propensity to aggregate. This SDS-soluble component of aggregates might represent the toxic species. The question thus also remains whether the toxic protein species must be identified in order to alleviate toxicity, or if any mHtt-lowering strategy would result in phenotypic improvements.

What's the best strategy to increase autophagy?

So it seems that the answers to Robert Pacifici's questions are as follows. Yes, autophagy can be stimulated. Yes, it appears to be "broken" in HD, but only at the point of cargo recognition within selective macroautophagy. And yes, it appears that stimulation of autophagy in HD would have benefits. But what's the best strategy for attacking mHtt? David Rubinsztein and many others have shown improvements in HD models and other biological systems with increased macroautophagy. Some

participants were concerned about negative effects that might arise from long-term autophagy stimulation. Depending on which step of autophagy might be broken, stimulation could have deleterious effects like autophagosome accumulation. For example, autophagosomes must reach the lysosome for degradation. This cargo trafficking requires movement along microtubule tracks using the dynein motor protein complex, which indeed interacts with Htt and may be functionally affected by polyglutamine expansion. Work in Alzheimer's disease (AD) shows that even though upregulated autophagy succeeds in clearing protein, autophagosomes accumulate after fusion with the lysosome, partly because of altered lysosomal pH. This appears not to be a problem in HD. Cuervo's work indicates that only cargo recognition in selective macroautophagy is disrupted in the experimental models her group studies. She surmises that with this distinct mHtt-induced defect, perhaps selective autophagy is lost but bulk autophagy is still working to clear mHtt at a basal level. Benefits could arise from either increasing bulk autophagy or repairing the lost selective autophagy. Rubinsztein summarized, "if you could do both, you might win. But it's a question of tractability. A practical consideration to manipulation of selective autophagy is that it would likely require a gene therapy approach, but stimulation of bulk autophagy could be achieved with existing FDA-approved drugs." Rubinsztein advocated for starting with stimulation of bulk autophagy, because Cuervo's work suggests that autophagosomes are working to clear mHtt, at least at some level. By increasing bulk clearance of mHtt, "one thing you're doing is getting rid of some of the Htt that is causing the selectivity problem, so you get a 'double whammy' effect…because mHtt is potentially blocking the access of other proteins to the phagosome." Rubinsztein proposed that another benefit might result from turning up nonselective autophagy: protection against caspase activation.

Simonsen added expressed her view that increasing non-selective autophagy alone wouldn't get rid of aggregates. To achieve this, one needs an autophagic receptor specifically targeted to recognize cargo. She believes that a better understanding of the molecular mechanisms underlying selective and nonselective autophagy will reveal the best way to target aggregated mHtt. Nukina also expressed his feeling that enhancing selective autophagy would be a more targeted approach, especially to clear ubiquitinated protein. Cuervo also shared her findings of several very different populations of lysosomes, which can be identified with various methods. One group is targeted very specifically by CMA, but those lysosomes can take up other materials as well. Autophagosomes tend to go to Group B, but if those are not available, they are less selective and will be taken up by Group A or C. Cuervo noted that, depending on the pathway targeted for stimulation, it may be important to consider the specificity of lysosomal targeting.

Clearly, safety concerns remain about constitutive upregulation of basal autophagy, because autophagy is a non-selective process with many downstream effects. Many currently used drugs induce autophagy and are taken for long periods with few side

effects. Work still remains to be done to answer the question, but participants by and large felt that it would be safe for several reasons. Indeed, increased autophagy appears to have diverse beneficial effects in many systems and increases lifespan in some animal models. Rubinsztein postulated that increased autophagy would not pose a risk to cells, because most macroautophagy substrates are long-half-life proteins, whereas most short-half-life proteins are cleared quickly by the UPS or by CMA. He has seen little effect on homeostasis of most proteins in the cell with induced autophagy. Cuervo pointed out that there is only one example in the literature of cell death brought on by even massive upregulation of autophagy: in the salivary gland of the developing fly. The beauty of the system is that it has tight feedback control mechanisms in place that prevent over-activation; other proteins can shut down the system. Rather than constitutive activation of autophagy, a therapy might also conceivably be effective with pulsatile stimulation. Bob Hughes envisioned a chemotherapy-like setting where patients might periodically receive high doses of an autophagy stimulator.

Key measurements: flux vs. level

Growing evidence suggests that upregulation of autophagy could protect cells by enhancing clearance of mHtt in various animals using a range of strategies, both pharmacologic and genetic. But human experiments are very difficult. Safe drugs exist that we could give patients now that might increase autophagy. But we lack markers of Htt and of autophagic activity in humans to show target engagement. Marc Hellerstein expressed his view that you "can't talk about anything until you can measure it. The critical measurement is of the flow of molecules through the autophagic pathway." He and others delineated two questions that need to be answered: defining the kinetics of the larger processes of autophagy itself, and the kinetics of turnover of huntingtin proteins, even in the absence of polyglutamine expansion. Both these processes are extremely dynamic and require flux measurement rather than a static snapshot. Measurements of molecular kinetics are much more sensitive and provide far more information than do measurements of levels of molecules. Static measurements by nature obscure small effects that could be revealed by kinetic measurements of a dynamic process, and they don't reflect the underlying changes that have created that small change in level. An elevation in the level of a marker of the autophagic machinery, such as LC3-II for example, could represent increased flux (increased formation of autophagosomes), reduced flux (reduced fusion and clearance of autophagosomes with lysosomes), or no change in flux (more autophagosomes, but no change in substrate uptake and throughput). Another barrier to flux measurements is lack of a clear Htt marker in human fluids. Several groups have looked and don't find robust Htt in cerebrospinal fluid (CSF).

Hellerstein described his new technique for metabolic measurement of autophagic flux. Though others have developed techniques to examine the kinetics of individual proteins, this technique allows one to see many proteins at once. The technique can be used in cell culture, animal models, or living people—any system that synthesizes

and processes proteins. The organism is fed a metabolic label, like heavy (deuterated) water, that gets incorporated into all synthesized proteins. The labeling protocol is carried out for the optimal time course for your protein of interest, whatever that may be. One then collects the fluid or tissue of interest and looks at the whole mixture of proteins (i.e. the intact proteome), or subdivides by selecting proteins from bands on a gel. Next, you trypsinize the proteins, generating many hundreds or thousands of peptide fragments. These fragments are then analyzed with a tandem mass spectrometer (MS). As peptides emerge from liquid chromatography (LC), the first MS analyzes the intact peptide, and the second MS analyzes ionic fragments of the peptide. The first MS measurement quantifies the pattern of metabolic label incorporation, while the second MS pieces together the amino acid (AA) sequence from the peptide fragments. Computer analysis uniquely identifies each peptide's protein of origin by comparison to the proteomics database. By mathematically analyzing the degree of perturbation and the pattern of labeling in each peptide, *in vivo* turnover rates are revealed for the hundreds or thousands of peptides that emerge, and for the proteins that they represent. In this case, proteins of interest could include proteins specific to HD such as aggregated or soluble mutant and wildtype Htt. Additionally, substrates of autophagy could be studied, including soluble, aggregated, or organelle-bound proteins. This *in vivo* labeling approach does not rely on overexpression or recombinant polyQ fragments. Because you're looking at native protein, it is relevant to all types and forms of Htt, and there are no confounding effects related to yield.

This newly developed technology for looking at the dynamics of the cellular proteome opens up to experimental testing several potentially important questions about Htt. One can now examine the two key questions of Htt flux and autophagic flux. For example, one can look at many autophagic protein substrates at once as an index of true autophagic activity, or look at many forms of the Htt protein. If different parts or fragments of a protein have different half-lives, one could see those. For most proteins, every proteolytic peptide fragment has the same half-life: that of the protein. But Htt fragments *in vivo* may have different half-lives and kinetics, perhaps depending on polyQ length. In terms of experimental design, if one is dealing with a long-lived protein like Htt, one can label for a long time period. Hellerstein has some data about the turnover of wildtype huntingtin in young mice revealing a half-life of about a week. One could even examine the potential differences in specific cell types, if the separate cell populations could be isolated. Spillover of proteins into the CSF or blood may allow the synthetic history, secretion, and degradation of relevant proteins to be assessed without the need for tissue sampling in humans.

Vellai also described his strategy to exploit the well known substrate of autophagy p62. Vellai is searching molecular libraries for specific small molecules that will bind p62. "Once we identify that, we can label it specifically and it can be used to see how it labels autophagic processes in a living human system."

Leslie Frank described her work trying to estimate the kinetics of soluble Htt in mouse neuronal cell lines using a fluorescence-based technique. She has been able to measure half-lives for exon-1 16Q, 44Q, and 76Q. Next, she is trying to estimate the half-life of the full-length Htt protein, beginning with 16Q and moving on to longer, toxic, mutant polyQ lengths. Once the system is set up for these kinetics measurements, they could use autophagy inhibitors and examine their effects as a way to indirectly quantify autophagy. Frank described the system. Mouse neuronal N2A cells over-expressed Htt exon-1 proteins after transfection using lipofectamine. The protein fragments contain a tetracysteine (TC) tag in the poly-proline-rich region, which serves as a marker of solubility. When unbound at the TC region, the tag is not fluorescent, but chemical staining leads to binding and fluorescence. By tracking the fluorescence of the protein in the cells over 24-48 hours, Frank has been able to calculate estimates of half-life. She saw that soluble Htt protein fragments turned over more quickly with a higher glutamine repeat number; that is, the higher the Q, the shorter the half-life. Accurate measurements of half-life of soluble Htt are very difficult to make. With 76Q, proteins aggregated very quickly, within eight hours. She limits the timeframe of experiments; once cells display aggregation they are not included in estimates of half-life. Tobin pointed out there are two sinks for soluble protein: aggregation and degradation. The future aim is to bring make this sort of measurement in the native system using knock-ins or some other approach.
Several people predicted that full-length Htt might present less interference from rapid aggregation, as it aggregates more slowly than do fragments. As an aside, Frank mentioned ongoing work at Johns Hopkins to develop MRI technology that would measure how the brain changes with HD progression.

Andrey Tsvetkov and Steve Finkbeiner have developed a microscopy system to view the dynamic process of huntingtin proteostasis in live neurons. Cortical and striatal neurons are isolated, cultured, and transfected with a protein consisting of wildtype or mutant Htt exon1 fused to a green photo-switchable protein called Dedra2, which turns red when illuminated with short-waved visible light. After optically labeling a pool of protein with this "pulse," Tsvetkov "chases" the decay of red fluorescence to measure the half-life of the mutant huntingtin protein fragment in any neuron type. Tsvetkov has found that the relative half-life of mHtt—rather than the expression level *per se*—is linked to cell viability. Neurons with a low mHtt expression level but containing long-half-life protein species are particularly vulnerable. Like Frank, Tsvetkov is measuring half-life of diffuse protein before inclusion bodies form, and he too has found that longer polyQ mutant proteins have a shorter half-life. When aggregates do form, the inclusions appear stable and some disappear rapidly, suggesting autophagic clearance. This optical pulse-chase technique can also provide a way to measure autophagic flux in live neurons. The team is developing a separate system that links the photo-switchable Dendra2 to LC3, a key autophagic

protein. Once the optical pulse switches Dendra2-LC3 to red, the protein fluoresces until it is degraded in the lysosome. Therefore, decay of fluorescence of Dendra2-LC3 can be used to measure the rate of flux through the autophagic pathway. This half-life can be manipulated with stimulators of autophagy. Fluorescent puncta indicating lipidation of LC3 (i.e. conversion from LC3-I to LC3-II) has served as the predominant visual marker of autophagy in non-neuronal cells. Evidence suggests that this LC3 conversion event differs in neurons compared to other cells. This microscopic technique measures flux of a fluorescent signal inherent in Dendra2, so labeling is independent of LC3's lipidation state and is visualized continuously. Tsvetkov and Finkbeiner believe that the Dendra2-LC3 system might reveal neuron-specific aspects of autophagy.

Additional tools:
Dimitri Krainc described a primary neuron cell culture system using cells from mice expressing full-length mHtt with 140-150Q. The cells develop significant toxicity compared to wildtype after ten days in culture (despite a mild phenotype seen in the mice) for which mHtt expression appears to be the only trigger. They are using the system to modulate autophagy genetically and pharmacologically.

Ivan Galimberti is using organotypic slice cultures in which brain slices can be grown for several months. Cortico-striatal slices are prepared at postnatal day 5-7. Although it's not an *in vivo* system, Galimberti said it's a good compromise to target medium spiny neurons (MSN) in a dish.

Suggested Experiments
At the conclusion of the workshop, Allan Tobin asked attendees to outline suggested experiments or research questions that CHDI should pursue. Their answers, summarized below, highlight some of the knowledge gaps that must be addressed.

David Rubinsztein proposed that the ideal would be to have a standardized readout for neurodegeneration itself, and secondly a readout for relevant processes that influence neurodegeneration, including autophagy, in the brain.

Tibor Vellai agreed that it will be important to monitor autophagy in a living system, and he proposes that p62 may be a good target to do this. He is also focused on efforts to model the process of autophagy itself. He had concerns about how much the process of autophagy can be safely stimulated. "If you hyper-activate it, that could be dangerous to kill its basal activity. We should find way to moderately activate the pathway, not over-activate it."

Zhenyu Yue raised the issue that we need to validate what we know from cell models in human patients using biomarkers. "We don't even know the status of *in vivo* autophagy in an HD patient. Can even boosted autophagy catch up with mHtt accumulation? If there is impairment in autophagy, can we repair it?" We don't

know how the autophagy process responds to accumulation of mHtt aggregates. Does mutant Htt create functional defects in autophagy, or does the gradual accumulation of aggregates eventually overwhelm the degradation system? These questions might be answered by taking a systems-biology approach, and specifically by looking at the process in peripheral tissues.

Ana Maria Cuervo recognizes that even boosted autophagy may not effectively catch up with accumulated aggregates. But genetic testing makes early intervention possible for HD. She also recognizes the concern with over-activating the autophagic system, but one possibility is that if you prevent accumulation of mHtt from an early age, you may avoid all the downstream consequences of toxic protein buildup, whatever that protein species might be. Further, she points out that multiple autophagic systems may be targeted, and the task at hand is to determine experimentally which one is best, and under which conditions, etc.

Andrey Tsvetkov will continue work to establish a photo-switchable system to study the dynamics of autophagy. The aim is to develop a system to study autophagy in live animals, to measure autophagy over time as the animals age, and to cross various mice. They will begin imaging the cortical layers, but sophisticated technology may allow for deep-brain imaging of the striatum and other areas, which might reveal cell-type-specific differences in autophagy dynamics even within neurons.

Ai Yamamoto recounted the experiments she carried out 11 years ago that showed that when inducible mHtt expression is halted in mice, protein aggregates are cleared from the brain and phenotype is improved. She has since been working to understand this result and to understand the role of macroautophagy in the brain in general. More specific questions that might lead to therapeutic targets include a better understanding of protein aggregation in neurotoxicity and in neurodegenerative diseases in general.

Ludovic Collin appreciates the importance of macroautophagy for HD and other neurodegenerative diseases, particularly in the role of clearing aggregate-prone proteins. Collin would favor the strategy of targeting selective macroautophagy over bulk autophagy because of the possibility for exploiting post-translational modifications that could target mHtt to autophagosomes specifically. Clearly we still lack the tools to study autophagy in brain, including a consensus about effective readouts of the process, which needs to be resolved.

Leslie Frank is most intrigued by the mystery of what the toxic protein species is in HD. “If we knew that, could target it more specifically.” In her work trying to measure the half-life of huntingtin, she encounters the important question of what constitutes soluble protein, and would like to see the peptide species of Htt and their kinetics more specifically defined. She looks forward to completing her live-cell

imaging of full-length Htt and comparing it to measurements of Htt fragment half-life.

Marc Hellerstein came to the workshop wanting to learn about the biology and biochemistry of autophagy with the preconception that we would need to find a way to measure autophagic flux. He has gained an appreciation of how interactive the autophagic systems are with one another and with the ubiquitin-proteasome degradation system (UPS). Hellerstein is impressed by how much we know about the autophagy system—what it is—and yet how little we know about its substrates—what it does. The questions he would most like to answer are, first, what are the kinetics of flux of substrates—including proteins and organelles—into and through autophagosomes. And second, what are the kinetics of flux of assembly and disassembly of mutant huntingtin aggregates. The dynamicity of exchange between aggregated and oligomeric mHtt, in particular, is currently unknown and has profound implications for strategies that target autophagy for the selective removal of mHtt from cells. He believes these measurements will be possible at least in cell systems using existing tools. A bigger but crucial hurdle will be to get to these measurements in humans, perhaps through measurements from cerebrospinal fluid (CSF) or non-neuronal cells.

Tamotsu Yoshimori underscored the need for a system to assay autophagy in humans. His lab continues their efforts to understand ways to upregulate autophagy, including screening small-molecule compounds. Like many others, Yoshimori believes we should know more about the way that huntingtin is processed by the autophagic systems for a more targeted therapeutic approach. His current focus on selective autophagy aimed at bacteria might provide insights to common mechanisms that also apply to aggregate-prone proteins. In terms of the safety concerns about stimulation of autophagy, he feels confident that at least within the cell, the multiple physiological autophagic processes are under strict control, preventing an "overshoot in autophagy" that might harm cells. Even with strong induction of autophagy, there appears to be a strict saturation level, and Yoshimori would like to understand the control mechanisms behind that control. Also, it may be very valuable to determine how expression of soluble recombinant polyQ rapidly induces autophagy.

Dimitri Krainc imagines an ideal situation in which mHtt could be made more degradable by post-translational modifications. Therapeutic benefits might arise from a slight upregulation of autophagy if aggregate-prone proteins were more accessible to degradation pathways. Krainc has focused on acetylation but is investigating how that modification "talks to ubiquitination and other modifications." Somehow, acetylation makes Htt more degradable, but it's not yet known how: does it interact with a recognized receptor, or is it indirect? He's also investigating how different forms of Htt (monomers, oligomers, acetylated protein) alter protein equilibrium, and which forms are more subject to degradation.

Mauro Piacentini would concentrate on modifying components of the Beclin 1 protein complex when thinking of therapeutics aimed at modulating autophagy because of its downstream position; it may provide a way to intervene with more specific effects. He continues to pursue his interest the relationships between autophagy, HD, and transglutaminase 2 (TG2). This avenue of research has provided hints that mHtt can be released to the outside of the cell for degradation. "Evolution has selected the easiest way to get rid of something: to put it in a vesicle and throw it out." We need to find the conditions that will induce this process.

Ivan Galimberti sees a real need to target neurons and the brain in studying autophagy. Unanswered questions include how autophagic flux is regulated in neurons, what cell-specific differences might be found in cortex vs. striatum, and how aggregated species of Htt are specifically targeted, including fragments vs. full-length protein. Next, he asked, which component of the pathway should be targeted for manipulation? He suggests an assay to screen for modulators of the autophagic pathway. To begin with, create a transgenic mouse line with neuronal expression of mCherry-GFP-LC3, and conduct time-lapse measurements of autophagic flux. Similarly, human embryonic stem (ES) cell lines expressing mCherry-GFP-LC3 could be used to assess autophagic flux in human cells, and both systems could then be used to carry out high-throughput screens for modulators of autophagic flux in neurons. Potentially, this could be extended to include HD-patient-derived induced pluripotent stem (IPS) cell lines. A secondary assay *in vivo* could determine if inducers that had effects in cells would also ameliorate an HD phenotype. As a genetic validation that increasing autophagy could ameliorate the effects of mHtt, Galimberti proposes the following experiment. Cross the Atg mouse lines currently used by Mizushima and Tanaka with HD mouse models to ask whether reduced neuronal autophagy accelerates the appearance of mHtt species—particularly aggregates—or speeds degeneration in cortex or striatum. One could optimize the experiment in terms of modeling human disease by using endogenous expression of a full-length mutant Htt protein, like the Hdh (CAG150) mouse.

Gill Bates would like to combine the approaches discussed at the workshop related to autophagy with approaches that target chaperone proteins directly. She also highlighted the need for a way to measure autophagic flux and the flux of Htt protein species *in vivo*, including the dynamics of proteins in and out of large aggregates and their degradation.

CHDI scientists raised the following questions to conclude the round-table suggestions and comments.

Doug Macdonald made a plea to participants to design experiments that will allow us to move easily into physiological settings: use full-length Htt with a physiologically relevant CAG length, and when possible, use neurons or do studies in

brain. Macdonald's question in moving experimental findings into therapeutic programs is determining the efficacy necessary to modulate disease course. Would a 10% reduction in mutant Htt be beneficial? We don' t know that yet. The temporal aspect is also critical to determining when intervention would come too late. With respect to autophagy specifically, how much gain or stimulation of the system do you need to get a benefit?

For Alex Kiselyov, a fundamental question is whether autophagy is dysregulated in HD, and whether these effects differ centrally and peripherally? Peripheral effects of mHtt directly on the autophagic process might lend clues to the disease state in the entire system.

Keith Elliston sees many challenges in understanding the role of autophagy in HD, and sees a need for a systems-level approach. His key questions include determining the role of the aggregates in toxicity, and whether stimulation of autophagy will have a beneficial impact.

Hyunsun Park sees one key question for CHDI's Autophagy group: is autophagy modulateable during pathogenesis? Although it's a loaded question, we will need to determine when, where, and how much stimulation might have beneficial effects, particularly in a chronic setting. Another fundamental unanswered question remains: we don't know what "aggregate" means and how the Htt protein dynamically moves in and out of these structures, with what effects.