

Hereditary Disease Foundation

Huntingtin Action: Up and Down the Axon

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Prepared by Tuan Hoang

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Summary:

On June 27 and 28, 1999, the Hereditary Disease Foundation sponsored a workshop on Huntington's disease titled "Up and Down the Axon." The goals were to bring together over a dozen scientific investigators with diverse backgrounds and skills and to facilitate the exchange of ideas and perspectives in an open forum. The main topic was: "What are the functions of the normal and mutant Huntingtin proteins in the cell?" To address this question, the workshop began with a discussion of the localization of the normal Huntingtin protein (Htt). Several possible theories on the normal function of the protein were then proposed based on the protein's subcellular localization and association with axonal transport proteins. The localization of the mutant Htt protein in various *in vivo* and *in vitro* models was also reviewed. Eventually, the possible mechanism of cell dysfunction and cell death was discussed. Consideration of the seeding of protein aggregates was followed by our focus on new potential techniques such as gene arrays that can be utilized to elucidate the pathway of neuronal failure in HD. The workshop concluded with the participants proposing new directions in research to advance the understanding of the disease and to expedite the development of an effective treatment.

Localization of the normal Huntingtin protein:

Steven Hersch began by sharing his observations from electron microscopic (EM) studies of brain tissue from mouse, rat, and post-mortem human brains. Using EM 48 antibodies, he found that the normal Huntingtin protein (Htt) was expressed mostly in neurons with a small amount in glia. Even within the same neuronal population, the expression was found to be heterogeneous. The protein was normally cytoplasmic; the protein was subcellularly localized throughout axons, dendrites, and perikaryon. No labeling was observed on the plasma membrane, nuclear membrane, Golgi, or smooth

endoplasmic reticulum. Some labeling was seen on the surface of the rough endoplasmic reticulum while much labeling was observed on the surface of microtubules and membrane-bound organelles, i.e., mitochondria and transport vesicles. In addition, Hersch cited Marian DiFiglia's immunoperoxidase study, which showed that Htt localized with clathrin-coated vesicles. However, Hersch pointed out that there is no reason to believe that this Htt-clathrin interaction is exclusive. Abigail Hackam asked if Htt appeared to be inserted into the membrane of the mitochondria. Hersch replied that the gold particles used in his EM studies would label the surface and would not determine if the protein was inserted into the membrane. Also, Stephen Davies inquired whether Htt had ever been observed inside the mitochondria; Hersch responded that he had not observed Htt inside the mitochondria; however, it was theoretically possible to detect with EM.

Function of the normal Huntingtin protein:

The function of the normal Huntingtin protein is unknown. Don Cleveland noted that since Htt is in so many places, this might mean that it is transported. Several participants agreed that since Htt is in the axon terminals, then it must have been transported. From its apparent association directly with HAP-1 and indirectly with microtubules, kinesin, and dynactin, it was proposed that the Huntingtin protein might be involved with either the transport of vesicles and/or mitochondria. Another possibility is that Htt may be associated with neurofilaments, intermediate filaments in neurons. Since the intermediate filament network is highly visible by light microscopic techniques in cultured fibroblasts, both Ethan Signer and Heather Durham asked whether Htt have been found to interact with vimentin, an intermediate filament protein in fibroblasts. No studies were referenced which could have provided strong evidence showing Htt interacting with either neurofilament or vimentin. However, Stephen Davies and Roy Dyer agreed that there is little vimentin in neuron,

suggesting that such protein interactions do not exist or are not involved in the pathogenesis. Kenneth Kosik added that only during development do neurons express vimentin.

Mitochondrial transport hypothesis:

Peter Hollenbeck proposed that Htt might be involved in the docking structure for mitochondria. Presently, there is some clear evidence for mitochondrial “docking.” Although no such docking protein has been identified, Letierrier’s lab has indirect evidence that microtubule-associated proteins (MAPs) may be involved. In an actively growing axon, the growth cone can be perceived as an ATP sink (high metabolic demand). Mitochondria have been observed to accumulate in the growth cone and to become stationary. The distribution of the mitochondria appears to be in a steep gradient, with far more mitochondria at the active tip than along the axon shaft. Conversely, when axonal growth stops, then the mitochondria are transported retrograde towards the cell body, spreading out into a relatively uniform distribution of roughly 1 mitochondrion per 20 μ m. There also appeared to be a recycling of mitochondria at the distal ends. Using voltage dyes, e.g. JC-1, to measure transmembrane voltage potential in mitochondria, high-acting mitochondria (new) were seen transported anterograde while low-acting mitochondria (old) were transported retrograde back to the cell body to be “recharged” or degraded. The motion was along either actin filaments or microtubules. When both actin & microtubules were blocked with drugs, no more motion was observed. Thus, is HD a result of the mitochondria’s failure to dock? If the mitochondria are not properly transported to areas of high ATP demand, can this induce a metabolic distress at the distal ends?

In corroboration, Hersch presented his preliminary result in a few lines of fibroblasts from juvenile, heterozygous, and homozygous HD patients. Using time-lapsed imaging with differential interference contrast, he and his colleagues measured the average rate of

mitochondrial movement over a set period of time. He found that the mitochondria moved faster and further as a negative function of their CAG repeats. In other words, the longer the CAG repeats, the more abnormal the mitochondrial movement. Presently he has expanded his experiment to 20-22 fibroblast lines with various pedigrees and is conducting the experiment blind. One caveat is that the fibroblasts are not as differentiated in subcellular compartments as neurons and that axonal transport of mitochondria can not be measured accurately. Thus, Hersch planned to use primary neuronal cultures for future experiments. Davies added that, in the Bates mice, neurite aggregates are surrounded by piled-up mitochondria. He wondered whether this “normal Htt function” can account for the abnormal mitochondrial clustering. Hersch countered that he sees few mitochondrial clusters around aggregates.

Localization of the mutant Huntingtin protein:

Steven Hersch recounted his observations from an EM study using EM 48 antibody. In HD brains with low grades (1-3), he found that more than 90% of aggregates were cytoplasmic, but not in the perikaryon. Instead, most were in dendrites and dendritic spines while some others were in axonal processes and terminals. He defined labeling of aggregates as a collection of gold particles representing Htt antibodies binding to those aggregates. The aggregates were mostly in spared neurons and sparsely in medium spiny neurons. Double labeling with Htt and diaphorase showed that 60% of these cells had nuclear aggregates. In HD brains with higher grades, Hersch observed that 20-30% aggregates were nuclear.

With light microscopy, Hersch and DiFiglia had independently found a difference in the distribution of the haloprotein of Htt. In normal controls, Htt was observed in axons/dendrites. Meanwhile, in HD neurons, Htt was found near the perikaryon. However, Hersch believed that the observation falls within normal

variability. Extrapolating from mouse to human HD, Davies expected only a few neurons (~2%) to have inclusions at any one time. Davies also cautioned that EM 48 antibodies did not delineate fibers and, thus, may not detect other diffuse Htt. Abigail Hackam asked if an EM experiment could be performed using double label N-terminal and full-length antibodies on the same section. Hersch replied that such an experiment could be performed using two different size gold particles but that it would be extremely difficult.

In vitro studies of mutant Huntingtin:

The participants also discussed several in vitro studies that attempted to determine the localization and aggregation of the mutant Htt protein. First, by varying the size of the Htt protein fragment in transient transfection experiments, Dyer observed that Htt appeared to enter the nucleus based on size and not by active transport. He found that the nuclear exclusion limit was 70 kDa. When a nuclear export sequence (NES) was added to the Huntingtin fragment, he observed no change in time course of cell death, which conflicts with results from Michael Greenberg's NES fusion protein study. In addition, Dyer found more death and at a faster rate than in Michael Greenberg's report. However, Dyer cautioned that the level of Htt expression could account for the differences as he had used lipofection instead of Greenberg's Ca_2PO_4 method. Abigail Hackam added that she saw no difference either in toxicity in 293T cells using an NES-Huntingtin fusion protein.

In addition, Dyer detailed a biochemical tissue extraction experiment using Triton X-100 (1%) + deoxycholate (0.5%) + SDS (0.1%). He used three successive extraction steps: S1 = supernatant from first extraction; S2 = supernatant from second extraction in same buffer; S3 = supernatant from re-extraction in SDS + urea.

In his normal control tissue, Dyer found similar levels of Htt in all three extracts, i.e., S1 ~ S2 ~ S3. Most protein fragments were larger than 100 kDa. In his HD tissue, he observed a

differential distribution of the protein extraction, i.e., S1 < S2 < S3. Again, most fragments were larger than 100 kDa, except for a prominent 40 kDa fragment. The Htt fragments were detected with antibody, 1C2 (specific for the polyglutamine tract near the N-terminus) but also with Chemicon antibody, 2168 (specific for an internal region near the C-terminus). Dyer was aware that his protein fragments could be from two different fragments with similar molecular weight.

Thus, he was micro-sequencing bands from S3 (least soluble) lysate. He had already found clathrin, a protein involved with the endocytic pathways, in his extracts. Using one Q42 brain from the Mayo Clinic and one grade-one HD brain from the McLean Bank (Harvard), he discovered differential sequestration of beta tubulin, kinesin and dynactin, proteins involved in the axonal transport pathways. Unfortunately, he could not confirm such an observation with two other grade-one HD brains. He attributed this to variability in the biochemical method. Signer suggested using HAIP to pull out the insoluble aggregates.

Evidence from transgenic/knock-in mice:

Localization of the mutant Htt protein in different mouse lines was also discussed at the workshop. Anne Young and Steve Hersch stated that in the MacDonald knock-in mice, Htt protein was found only in the nuclei, where many very small aggregates formed. There were many more in the striatum than the cortex. The Hayden mice had less nuclear Htt and fewer inclusions. On the other hand, the Borchelt mice had many more inclusions in the cortex than the striatum. Davies noted that in the Bates mice (R6/2 line), he had found prominent nuclear inclusions in the majority of the cells, both neuronal and glial. His belief was that the longer repeat length caused more nuclear translocation but not necessarily more aggregate formation of Htt.

Davies stated that he was presently raising antibodies against purified inclusions. The new antibodies did not see anything in immunostudies with normal brain or with protein

extracts. However, the antibodies do have much more staining in transgenic mice and human HD than with any other known antibodies. The new antibodies showed much more staining of nuclei and dendrites but did not stain inclusions except for a dark annulus at the edge, i.e., the inclusions that were labeled by anti-proteasome antibodies were not revealed. In addition, they did recognize Htt protein aggregates formed *in vitro* but they did not recognize Htt protein on Western blots. When the poly-Proline tract was deleted, Htt inclusions were shown to be more densely packed, which could alter their conformation and reactivity with antibodies.

Possible gain of function of mutant Huntingtin protein:

Allan Tobin posed the question, “Is Huntington’s disease a disease of interneuronal communication or intraneuronal havoc?” On one hand, does striatal sensitivity result from high level of glutamatergic input? Zachary Mainen felt that this was unlikely because receptors could be downregulated when overstimulated. James Eberwine volunteered to defend the “interneuronal communication scenario” by stating that presynaptic input could modulate dendritic protein synthesis, e.g., stimulate production of nucleating protein.

Tobin was quick to emphasize that, by determining the exact mechanism involved, a therapeutic agent could be developed which could effectively target the dysfunction. To address the question, several points were clarified.

Question One: Which comes first: neuronal dysfunction or neurodegeneration? Hackam and Hersch presented their observations on the Hayden YAC mice. At six months, the Q78 low-expression mice were found to be deficient in LTP and showed elevated Ca^{2+} transient baseline. At 12 months, the mice displayed neurodegeneration, mostly in the striatum. The cell death was not TUNEL-positive; the same striatal cell types were spared as in human HD. In conclusion, LTP changes were independent of cell

death, cellular dysfunction occurred before cell death; much cell death occurred before aggregate formation and before motor behavioral changes. In contrast, Davies pointed out that in the Bates mice, aggregation was observed at 4 weeks, thus before the appearance of a phenotype. However, the phenotype was seen before cell death at 14 weeks. Furthermore, he had determined that most cell deaths were observed in the cells of the anterior cingulate cortex and the Purkinje cells of the cerebellum. Tobin mentioned the fact that the normal expression of Htt was relatively high in pyramidal neurons of the cerebellum and cortex and asked whether these same cells died in the YAC mouse model? Hackam and Hersch replied that they had not specifically checked for it. Davies was quick to point out that CA1 pyramidal cells were earliest to have aggregates in the Bates’ mice but did not die. Thus, aggregation may or may not be involved in the pathogenesis, but may be a marker of abnormal disposal of huntingtin.

Maidment and Tobin referenced Michael Levine’s cell swelling study, which found the neurons of the Bates’ mice to have increased NMDA sensitivity. In addition, Nigel recalled Marie-Francoise Chesselet’s *in situ* study, which showed that the enkephalin mRNA was down in the Bates’ mice compared to control. Maidment also presented his preliminary microdialysis results showing no change in enkephalin levels, despite the mRNA changes.

Question Two: Is the phenotype from cell death or from accumulated dysfunction? Is early cell death bad since these cells are dysfunctional (diseased)? Would the brain work better if dysfunctional cells were not preserved but allowed to be eliminated? Don Cleveland and Allan Tobin posed these questions to initiate the discussion. Tobin stated that pallidotomy for Parkinson’s disease was based on such a premise of disconnecting the circuit of dysfunction. Kosik recalled that “Jean Pol” had previously shown that although 50% of the cells died, there were the remaining 50% of the cells that were spared.

Thus, emphasis should be placed on examining the remaining cells and determining the factors that protected them. Maidment concurred that the spared neurons may be the ones that could put up a defense to the insult. Cleveland suggested that one factor may be anti-cell death molecules, such as those of the BCL-2 family. Perhaps, spared neurons induced expression of BCL-2 allowing mutant Htt to be more “happy.” Tobin referenced the study, which crossed “leacher” mice with BCL-2 mice. The progeny were found to have similar degeneration of the Purkinje cells as the leacher mice, but noted lack of degeneration of susceptible neurons elsewhere. One interpretation of this result was that the investigators were not able to stop suicide but they were able to stop murder.

Hersch then proceeded to ask, “Who is the murderer?” Huntingtin? He reminded everyone that in humans diaphorase-positive neurons make almost no Htt but have many inclusions! Weissman proposed that Htt expression should be turned off in the adult neuron. Signer and Wexler responded by citing a study recently performed by Ai Yamamoto and Rene Hen using a tetracycline-off promoter linked to exon 1 of the IT15 gene allowing for conditional expression of the Htt fragment. Mice expressing the mutant Htt showed inclusions in the striatum and cortex after four to five months of age. If the expression of the protein were allowed to continue, the symptoms became progressively worse. If the mutant Htt expression were turned off, however, the mice improved. Analysis of these mice without Htt expression showed relatively less aggregation. In addition, D1 dopamine receptors were found to be upregulated; D2 dopamine receptors were still found to be lower than normal.

Question Three: Is apoptosis directly involved in the pathogenesis? Stephen Davies responded by stating that in the Bates mice, the TUNEL-positive apoptotic cells during development (5 days) appeared morphologically different to those cells that died at 12 weeks (not TUNEL-positive).

Hackam mentioned the Friedlander paper, which argued that caspase was involved in the mechanism of cell death. Signer referenced a study where mice expressing mutant Htt (dysfunctional) were crossed with mice not expressing caspase (knock-out). The result of the experiment was that the progeny mice had improved motor behavior. Davies noted that four different antibodies to FADD, an adaptor protein to caspase 8, do not stain inclusions in Bates mice or human HD. But he did concede that FADD is also hard to localize in normal brain. Kosik referenced the Siegel paper that discussed caspase activation and death effector domain (DED) filaments.

Heather Durham presented her results using a primary culture model of ALS, in which mutant SOD-1 cDNAs were introduced into motor neurons of dissociated spinal cord cultures. Treatment with caspase inhibitors had no effect on the time course of death of these motor neurons. Co-expression of bcl-2 had only a minor result on formation of SOD-1 cytoplasmic aggregates. On the other hand, the calcium binding protein calbindin, heat shock proteins and glutamate receptor antagonists had profound effects, both preventing formation of aggregates preserving viability. This meant that therapies need to target upstream effects that lead to cell death. Wexler reaffirmed the need to prevent neuronal dysfunction instead of blindly blocking the end-stage of death of dysfunctional cells.

Lessons from tau research:

Kenneth Kosik stated that ubiquitin hydrolase may play a very important role. One example given was UCHL-1 (PGP 9.5) ubiquitin hydrolase mutant which produces Lewy bodies and tau tangles. Normal tau is a very unfolded protein (very low-helix content). In addition, it is very soluble (boiling, 0.75 M NaCl, ClO₄) and denatured in vivo. It contains 3 or 4 repeats in C-terminus arising as alternate splice forms. The lysines located in these repeats interact with glutamic acids in microtubules. Tau protein forms the core structure of fibrils.

Kosik further explained the seeding of tau assembly. He referenced Eckhardt and Eva Mandelkern who proposed that intermolecular disulfide bonds seed the aggregation. Experiments have shown that tau dimerizes in early stages of assembly. Heparin or RNA can also seed the tau assembly by setting up the charged areas.

Tobin asked what diseases could be caused by mutation in the tau protein. Kosik answered that mutation of the tau protein has been found in cases of frontal lobe dementia. In this disease, the mutation occurs in either the coding region or intron resulting in tau fibrillization. Intron mutations affect splicing and cause an increase of 4-repeat forms.

Cleveland asked if the phosphorylation of tau occurred before or after fibril formation. Kosik replied that this was unknown. Young recalled the work of Brad Hyman who triple stained for neurofilament, amyloid, and Paired Helical Filament (PHF). He observed an axon entering an amyloid plaque where it became wiggly. At that point, PHF staining could be observed. It then straightened out again upon exiting the plaque. If there was a mutation in tau, then it was expected that fibrils would be internal.

On the other hand, if there were a mutation in APP, then this would set up fibrils externally. Anne Young further stated that tau knock-out mice were alive but did not show any behavioral and morphological changes. Also, overexpression of tau in transgenic mice also did not induce any phenotype. Thus, one interpretation was that tau mutation resulted in gain of function.

Autophagic pathway:

Hollenbeck proposed that we should consider the involvement of the autophagic ("dumb gulp") degradative pathway in Huntington's disease. As a brief overview, the multilamellar membrane from the rough endoplasmic reticulum condenses into vacuoles. It then fuses with lysosomes for degradation. Thus neurons can clean up the entire cytoplasm in 3 to 4 days. One question was whether a lot of

the aggregates can be cleaned through this process. Furthermore, are there Huntingtin proteins in lipofuscin? Hersch responded that his EM studies have not seen Htt inside lysosomes. One caveat is that maybe the antibodies could not penetrate the membrane. Davies added that neurons in Bates R6/2 mice were full of lipofuscin. He also saw this vacuolization in rapid onset in patients with Parkinson's disease. Hersch noted that HD neurons had lipofuscin too. However, he saw no vacuoles in Borchelt or Hayden mice. Hackam has bred Hayden YAC mice onto a null background and found that mice with expanded polyQ had vacuoles in their testes, while mice with short polyQ did not.

Employment of microarrays in HD research:

Anne Young presented preliminary results on the use of Affymetrix's chips (6500 genes) on Bates R6/2 mice at 12 weeks of age. She found changes in 71 genes; most were decreases in expression with a handful of increases. Most of the affected genes were involved in signal transduction (transcription, G-protein, Ca²⁺, enkephalin, D1 and D2 receptors). No cell death genes were affected. She is presently conducting experiments on six-week-old mice.

James Eberwine proposed to form a consortium for array work in which members could share probes, data, tissue (e.g., from Wexler's Venezuela project). As much clinical information as possible should be provided. The raw data could then be placed on a webpage so people could apply their own analysis techniques. Presently, the commercially available arrays are from Clontech, Research Genetics, and Synteni (InCyte). He cautioned that the secondary screens were the most critical and that the gene of interest may not be represented in the arrays which only contain high abundance transcripts. Kosik mentioned that striatal-specific arrays (human and mouse) would be extremely valuable. He believed that to understand selective vulnerability (1) striatum must be special (make striatum-specific array) and (2) selection in neuronal lines in culture (transfect in library, identify clones that

rescue toxicity). Eberwine and Tobin stated that the possible sources of striatal libraries for array construction were Jim Boulter, Hans Lehrach, Leroy Hood, Nat Heintz, Glenn Evans, and Bento Soares.

Concluding remarks:

The universal conclusion among the participants was the importance of understanding the role of the normal Huntingtin protein. Weissman and Hackam emphasized that detailed investigation of the Htt subcellular localization and translocation should be conducted. Hackam also proposed to determine if any post-transcriptional modification of the Htt protein occurs normally. Both Hoang and Dyer felt that further research into the fragmentation/ cleavage of the protein should be performed because it might provide clues for the mechanism of the cellular dysfunction. One direction would be to focus on known protein interactors. Dyer suggested that MAP kinase pathway might be involved. Mainen recommended that *in vivo* recordings, e.g., LTP induction, should be pursued to extensively characterize the nature of the dysfunction. Davies felt that electro- physiology in the Hayden mouse would be fruitful because of its neuropathology. Furthermore, Kosik advocated the research into the mechanism responsible for the selective neuronal vulnerability. Mainen proposed that the physiology of striatal cell should be extensively documented prior to their death. On the other hand, Hersch suggested using single cell profiles for differential vulnerability. Kosik wondered if there was a seeding reaction required for aggregation. Davies and Hersch believed that it was important to determine once and for all whether aggregation was a helpful defensive mechanism of the cell or part of the toxic process.

Davies stated that it was possible to study the importance of seeding in aggregation using an *in vitro* aggregation assay. Weissman believed that the role of caspases should be investigated in more detail. He mentioned that it was possible to develop a degradation assay in yeast. Kosik and Hollenbeck argued that therapeutic drug

screening, e.g., caspase inhibition, should be performed, regardless of our lack of understanding of the underlying mechanism of the disease. In more detail, Hollenbeck proposed utilizing “profiling” and combinatorial chemistry for leads. Kosik added that cell based screening assay could be employed as well as chip arrays (including striatal array). Davies and Hackam concurred that arrays would be interesting, especially since it may find “what’s not expected.. Durham insisted that good primary culture models should be utilized for secondary screens.