

# Hereditary Disease Foundation

New News on HD

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Prepared by Andrew Lieberman

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The Hereditary Disease Foundation's one-day workshop entitled "New News on HD" preceded the 1999 meeting of the American Society of Human Genetics in San Francisco. Discussion focused on animal models of Huntington's Disease and other CAG repeat disorders, the normal function of huntingtin and its effect on transcription, and the possible role of the proteasome and ubiquitin pathway in mediating disease.

### **1. Animal Models**

A table of the available mouse models of polyglutamine disorders was distributed, and additions/corrections were noted by the meeting's participants. Danilo Tagle indicated that the expression levels in his full-length huntingtin 48 repeat mice are five times endogenous, while levels in his full-length 89 repeat mice are two to three times endogenous. The behavioral characteristics of the heterozygous mice include weakness at 8 weeks as measured by the inverted screen test and vertical pole defect, hyperactivity at 12 to 32 weeks, and hypoactivity at 40 to 60 weeks. Most animals are dead by 68 weeks. Homozygous mice have a shortened life span by about two months. The pathology of the 48 and 89 repeat lines is similar, with cell loss/neurodegeneration occurring in the hypoactive period. In both cases, the striatum and layers 5 and 6 of the cortex are affected. Intranuclear inclusions are observed in animals starting about 12 weeks. They occur in less than 1% of the neuronal nuclei. Most aggregates that are observed occur in the perinuclear region or in neurites. Transgenic mice expressing a truncated version of the expanded repeat protein (exons 1-3) exhibit similar behavioral changes.

Christopher Ross noted that his DRPLA mice exhibit late neuronal loss (up to 30 – 40%) in the dentate nucleus of the cerebellum. He also mentioned that a new DRPLA model from Shoji Tsuji's laboratory is being presented at the ASHG meeting. That model was reportedly made using a cosmid containing the entire DRPLA gene. One line containing 129 CAG repeats shows disease onset at 4 to 6 weeks, with pathology reportedly occurring in the regions affected by this disease in humans.

Diane Merry reported on her work to develop a mouse model for SBMA. She has used two different promoters to drive the expression of truncated forms of the androgen receptor that contain up to 112 glutamines. These truncated constructs contain about 10% of the total protein (100 amino acids in addition to the polyglutamine), and lack the DNA-binding and ligand-binding domains. When using the PrP promoter, severely affected lines show transgene expression in all neurons (magnitudes greater than endogenous levels) with the development of widespread inclusions that are exclusively intranuclear by 6 weeks. She has eight PrP lines derived from different founders, with varying degrees of disease severity. The most severely affected line shows a phenotype by 3 weeks of age with hindlimb gait abnormality, resting tremor, sudden shudders, and handling-induced seizures. These animals typically die by six weeks. Lines with late onset disease begin to show a phenotype around eight to nine months with decreased activity, hindlimb foot claspings, and slow progression to occasional tremors. Pathologic examination has revealed no evidence of neuronal loss in the brain, no abnormalities of peripheral nerve, and no evidence of neurogenic atrophy in skeletal muscle. Males and females of all lines show decreased fertility.

The neurofilament light chain promoter was also used to drive expression of the expanded repeat androgen receptor transgene. These mice have high levels of transgene expression in cortical neurons and in motor neurons of the brainstem and spinal cord. Low levels of pan-neuronal expression are also achieved. NF-L promoter mice exhibit a phenotype characterized by decreased activity, hindlimb claspings, spastic gait, hindlimb muscle weakness, and perineal muscle weakness. The phenotype is progressive and first evident around 8 months, and apparently reflects involvement of both upper and lower motor neurons. Inclusions are present in neurons of the cortex, and motor neurons of the spinal cord and brainstem. There is no evidence of neurogenic atrophy in skeletal muscle or axon loss in ventral roots.

Jean Louis Mandel described his laboratory's efforts to make an animal model for SCA7. They have used three different promoters: PDGF to get

widespread neuronal expression, rhodopsin to target photoreceptors, and Pcp2 to compare with SCA1 mice. They have observed nuclear inclusions in all lines, with loss of photoreceptors confirmed by electroretinogram. PDGF animals show a neurological phenotype with trembling/seizures.

When assessing the value of transgenic animals, Ethan Signer cautioned about the “hidden variables” in the models currently available. These include (1) the fact that neuronal dysfunction may be more relevant to disease than neuronal death. Also, (2) cellular dysfunction/death may be due to murder rather than suicide. That is, the mutation may exert its primary effect on a cell other than the striatal neuron (in HD) with the resulting loss of trophic support causing the clinical and pathologic changes characteristic of the disease. (3) Many of the transgenic models use constitutively active promoters, such as CMV or PrP, so that developmentally regulated expression of the transgene is not achieved. (4) Transgene expression levels are often far above those of the endogenous protein, thereby complicating our interpretation of these animals. Finally, (5) chromosomal location of the transgene may be important.

Leslie Thompson raised the issue of strain background differences in mouse models. She noted recent work by Oswald Steward on mouse models of epilepsy. Animals are injected in the hippocampus with kainic acid, an excitotoxin, to induce seizures. Though all strains develop seizures in this protocol, the neuropathology is varied. FVB-N mice show evidence of cell death in response to kainic acid, while C57Bl6 mice do not. In this system, p53 knockout mice are also resistant to excitotoxic cell death in a strain-dependent manner. After performing a large series of crosses, Steward reportedly believes this protective effect is due to a single locus plus possibly a single modifier gene, which he hopes to identify. Similarly, Diane Merry noted that a mouse model of Parkinson’s disease created by David Borchelt using an SOD mutation had a phenotype on a mixed C57Bl6/C3H F1 background. These animals lost their phenotype when backcrossed onto an inbred C57Bl6 background. Huda Zoghbi reported a similar

influence of mouse strain on the phenotype of SCA1 transgenics. It appears that the Bl6 background may provide protection against neurodegeneration in various model systems.

A related series of experiments by Patrik Brundin were discussed. He has injected quinolinic acid into the striatum of the Bates R6/1 mice and nontransgenic littermates. Neurons from the HD transgenic mice were more resistant to excitotoxic cell death than were neurons from wildtype mice. There are no known changes in NMDA receptor levels or ligand binding in the transgenic animals. It was suggested that chronic low-level stress could result in protection from cell death in these animals. It was also suggested that there may be a disconnection between the cortex and striatum, which results in neuroprotection.

The tet-off, huntingtin exon 1 transgenics made by Ai Yamamoto were also discussed. In this model, in vivo inducible expression of expanded repeat exon 1 (98Q) was achieved by crossing TetO-exon 1 transgenics with mice expressing tTA driven by the CamKII promoter. Transgene expression results in behavioral changes including foot claspings, decreased expression levels of D<sub>1</sub> and D<sub>2</sub> receptors, and the appearance of intranuclear aggregates. When the transgene is turned off, the behavioral changes and intranuclear aggregates go away (fully in striatum, partially in cortex). These findings suggest that the phenotype of this mouse is due to neuronal dysfunction rather than neuronal death.

Christopher Ross noted that the functional impairment of HD patients appears to correlate strongly with neuronal loss. Neuronal loss was measured by both the Vonsattel pathologic grading system and MRI studies of volume loss in the caudate and putamen. Functional impairment of HD patients was assessed by measurement of activities of daily living and a motor impairment score, which incorporates rigidity and incoordination. In contrast, he noted that chorea does not correlate well with pathologic or MRI grading, and may in fact reflect neuronal dysfunction.

The importance of modifying genes was also touched upon. Christopher Ross noted that repeat length only partially explains age of onset in

patients with HD, accounting for 50-60% of the influence on this variable. Modifiers may account for much of the remaining effect. He hopes to identify these modifying genes by conducting a genome wide scan, in collaboration with Marcy MacDonald. Nancy Wexler indicated that there are also plans to use DNA from Venezuelan families to look for modifiers by a genome wide scan. She additionally indicated that she could provide material to look at effects of candidate genes.

## 2. Normal Function

Christopher Karlovich has identified the *Drosophila* ortholog of huntingtin by mining the EST database. He has now isolated the entire *Drosophila* gene, which encodes a 3500 amino acid protein that is similar to mammalian huntingtin in three regions. These regions of similarity are three to four hundred amino acids long, show 25% identity and 40% similarity to the mammalian protein, and may define functionally important domains. No other functional motifs were identified in the *Drosophila* gene. No caspase cleavage sites or polyglutamine/polyproline stretches were present, implying that these may not be needed for normal function. The gene organization is not conserved between *Drosophila* and human. *Drosophila* huntingtin is encoded by 29 exons, while the human gene has 67. Intron/exon structure is also not conserved in the three regions of amino acid similarity. In situ analysis was attempted, but failed to identify a specific signal likely due to very low expression levels. He plans to mutate the *Drosophila* gene to study its normal function. He also hopes to study the functions of HAP1, HIP1, and HIP2 in this system, since *Drosophila* orthologs of these genes have been already identified.

Robert Hughes suggested that the issue of gain versus loss of function in HD may be overstated. Evidence showing that expanded repeat huntingtin can recruit and potentially deplete the cell of the wild-type allele could suggest an underlying loss of function. In this case, understanding the normal function of huntingtin and other CAG repeat disease genes could provide insights into these diseases, especially if commonalities of function emerge. Christopher Ross noted that the wild-type androgen receptor and possibly huntingtin are

neuroprotective. Is this true for other disease protein? Are they all proteolytically cleaved? Do they all have functional NLSs? Are they found in the nucleus? Are there other aspects of their function in common?

Russ Margolis pointed out that there are two known proteins that normally have 38 to 40 consecutive glutamines and are nonpathogenic. These are TATA binding protein and so-called glutamine-rich protein, recently identified by his group. Glutamine-rich protein is about 300 amino acids long and consists of 30% glutamine, including a stretch of 40 consecutive glutamines that is broken by eight amino acids and then followed by another 10 glutamines. It is expressed at quite low levels, which may account for the fact that it is nontoxic. Overexpression of the normal protein in both neuroblastoma and COS cells results in cytoplasmic aggregation. The size of the aggregates increases as the glutamine stretch is lengthened. Overexpression of either form (normal with 40 consecutive glutamines, and expanded with 79 consecutive glutamines) results in moderate toxicity, as measured by direct cell counts after cotransfection with a GFP expression vector.

There was a brief discussion as to whether nuclear localization was necessary for neurodegeneration in HD. Albert La Spada noted that neuropil aggregates occur in axons and axon terminals where excitotoxicity may be mediating its effect. He questioned whether nuclear localization was key to the molecular cascade that mediates the disease. Others cited previously published work on huntingtin in cell culture by Frederick Saudou and on SCA1 in transgenic mice by Harry Orr and Huda Zoghbi supporting the importance of nuclear localization. Robert Hughes noted his work in yeast, and Henry Paulson's work on MJD showing that if one expresses proteins with normal repeat lengths in the nucleus, these proteins are prone to aggregation. This suggests that the nuclear environment favors aggregate formation. He suggested that if proteolysis of huntingtin is important, it may exert its effect by trimming the protein to a size that can freely diffuse into the nucleus and aggregate, even without an NLS. Christopher Ross noted that putative nuclear localization signals in huntingtin have been

previously identified, several near the N-terminus and one near residue 1100, but none have been shown to be functionally important. His laboratory is currently making transgenic mice expressing truncated huntingtin with an attached nuclear export signal in hopes of evaluating this question. He noted that their transgene contains the NES at the C-terminus of the molecule, and it appears to be cleaved off *in vivo*. He plans to generate new animals with an N-terminal NES to more definitively address this question. Similarly, Josephine Dorsman is using a *C. elegans* model to address this question, with constructs containing either an NLS or NES.

### 3. Transcription

Leslie Thompson and Joan Steffan shared the results of their recent studies on the role of huntingtin as a transcriptional repressor. They had observed that the structure of huntingtin resembles a transcription factor, with a glutamine-rich domain followed by a proline-rich domain. P53, a tumor suppressor protein and a transcription factor, contains a similar structure, with an amino-terminal acidic activation domain followed by a proline-rich domain. The proline-rich region of p53 contains five src homology domain interacting sites or SH3 motifs, encoded by amino acids PXXP. Huntingtin also contains a proline-rich domain with several potential SH3 motifs. Based on these structural similarities, they wondered whether huntingtin and p53 may interact and/or function similarly. They also noted that all of the triplet repeat disease genes except MJD have a src homology domain interacting site within a proline-rich region that is near the polyglutamine repeat.

Their initial experiments utilized *E. coli*-produced GST-fusion proteins that contained huntingtin with 25Q and the proline domain, or 51Q. These proteins were attached to glutathione agarose beads, and were used to show an interaction with radiolabeled p53. This interaction was not dependent on Q length, but was dependent on the presence of the proline-rich region. The interaction was also dependent upon the C-terminal domain of p53, as truncated constructs did not bind huntingtin. This interaction did not effect DNA binding by p53 as measured by gel shift assay.

They next performed co-immunoprecipitation. Initial attempts were unsuccessful since an antibody against the CAG repeat from Eric Wanker immunoprecipitated p53. Similarly, an antibody against p53 immunoprecipitated an N-terminal fragment of huntingtin. Co-immunoprecipitation in transiently transfected cells using truncated huntingtin with a C-terminal GFP tag and anti-GFP antibodies showed an interaction with p53. They also did GST pull downs in cell culture, and found the interaction between huntingtin and p53 was not dependent on the proline rich region, and occurred with both normal (25) and expanded polyQ (104) tracts. P53 was also identified in aggregates from a cell culture system developed in David Housman's laboratory that utilizes HEK293 cells.

They next looked to see if exon 1 of huntingtin could affect p53-mediated transcription. To do this, they used the p21/Waf1 promoter (activated by p53) driving a luciferase reporter. They found that a huntingtin construct encoding 104 glutamines and the polyproline domain decreased expression of the reporter, while normal repeat huntingtin or expanded repeat constructs lacking the proline domain had no effect. SAOS 2 cells, osteosarcoma cells that lack p53, were transfected with MDR 1 promoter-luciferase constructs to test whether huntingtin acts similarly to p53 to repress transcription. Huntingtin with 104 glutamines, and to a lesser extent 25 glutamines, blocked reporter expression. Since the p21/Waf1 promoter requires CBP/p300 for activation, and David Housman's laboratory recently found CBP in aggregates in their cell culture model of HD, they next looked for an interaction between huntingtin and CBP. Using a truncated form of expanded repeat huntingtin fused to GST, they demonstrated an interaction between the expanded polyQ protein and full-length CBP.

Cathy Hartog and Lesley Jones have also found that huntingtin can act as a transcriptional repressor. They found that a huntingtin-GAL4 fusion decreased the activity of a reporter construct by 90%. Their reporter consists of the tk promoter with five GAL4 binding sites upstream, driving expression of luciferase. The effect of huntingtin as a repressor was not repeat length dependent. They have also found that

transient expression of N-CoR in HEK293 cells, which seem to lack endogenous N-CoR, results in the transport of normal, endogenous huntingtin from the cytoplasm to discrete regions of the nucleus. They do not yet know whether the nuclear huntingtin has been processed.

Jon Wood and Christopher Ross have similar findings in their studies of atrophin 1, the protein mutated in DRPLA. They have looked for proteins that interact with atrophin 1 by yeast-two hybrid. The interacting protein they identified is ETO, or "eight twenty-one" (also called mtg8). ETO functions as part of the transcription repression machinery and also binds directly to N-CoR. Atrophin 1 was found to co-localize with histone deacetylase, and in transient transfection assays represses the expression of a reporter construct independent of repeat length.

Alexander McCampbell and Kenneth Fischbeck have studied the interaction of CBP with the androgen receptor, the gene mutated in SBMA, based on findings that CBP interacts with the normal androgen receptor. They found that CBP co-localizes with truncated, expanded repeat androgen receptor in nuclear aggregates in a motor neuron-neuroblastoma hybrid cell line. Recruitment of CBP to aggregates was also found in transgenic mice expressing a truncated form of expanded repeat androgen receptor made by Diane Merry. In transgenic mice, about half of all aggregates are immunoreactive for CBP. In collaboration with Gen Sobue, they also found CBP in aggregates in patient tissue. To look for a functional effect of CBP recruitment to the aggregates, HEK293T cells were transiently transfected with truncated forms of the normal or expanded repeat androgen receptor, with or without CBP. Cell survival was measured by a metabolic assay, MTT. They found that coexpression of CBP and expanded repeat androgen receptor blocked the toxic effect of expanded repeat androgen receptor. Expression of CBP alone, or with normal or expanded repeat androgen receptor, also exerted a significant proliferative/survival effect on their cells.

Ethan Signer noted that several laboratories have data suggesting that p53 may have deleterious effects in models of polyglutamine disease, while CBP and AKT may exert positive

effects. He pointed out that lithium is known to increase CBP and AKT activity, and decrease p53 activity. He suggested that it may be worth evaluating lithium as a potential therapeutic. Christopher Ross stated that lithium has been given to some HD patients to treat bipolar disorder and he will look to see what their longitudinal course has been, although it will be too few to draw many conclusions.

#### **4. Proteasome Function**

Proteasome function and dysfunction has been the focus of work in several laboratories. Christopher Cummings and Huda Zoghbi crossed SCA1 expanded repeat transgenics with knockout mice lacking ubiquitin E3 ligase, the gene mutated in Angelman's syndrome. E3 ligase is normally expressed in Purkinje cells at high levels, and the knockouts are a Purkinje cell specific null mutant. Knockout of this enzyme in mice results in impaired rotorod performance without morphologic changes in Purkinje cells. When SCA1 transgenics are crossed onto the knockout background, the resulting mouse develops cerebellar degenerative disease that is morphologically similar to, but more severe (earlier onset) than that seen in the SCA1 transgenics. Although the disease is more severe, intranuclear aggregates are far less common in these mice, identified in about 10% of all Purkinje cells, suggesting that ubiquitination is important for aggregate formation in vivo.

Proteasome components and chaperones have been identified in the inclusions seen in expanded repeat androgen receptor transgenic mice generated by Diane Merry. She also reported preliminary data indicating that in vitro translated expanded repeat androgen receptor protein has a longer half-life than does normal repeat protein, suggesting that mutant androgen receptor is not degraded as efficiently by the proteasome. Huda Zoghbi noted that she has similar data on SCA1.

Robert Hughes studies proteasome function in yeast. He has expressed an N-terminal fragment of huntingtin that contains amino acids 1-170. An expanded repeat protein containing 75 glutamines, an in-frame deletion of the polyproline domain, and an NLS produced no toxicity. He then disrupted proteasome function either with canavanine, an amino acid analog, or

cadmium, a heavy metal. When the amino terminal fragment of expanded repeat huntingtin was expressed in the presence of either of these agents, he noted ten fold increased sensitivity to proteasome disruption as measured by cell death. When a similar huntingtin construct encoding a normal number of glutamines was expressed in the nucleus, he observed inclusions but no increased canavanine sensitivity.

Bill Richards raised the question whether aggregates in polyglutamine disease and in other neurodegenerative disease could act as ubiquitin sinks and therefore effect overall protein turnover.

### **5. Other Questions and Model Systems**

Discussion briefly touched on a number of questions that are being studied by several laboratories. Albert La Spada is interested in how genomic context influences whether a CAG repeat will undergo expansion. He has created YAC transgenic mice containing the androgen receptor gene with 45 CAG repeats. He found that these mice show repeat length instability, while cDNA transgenic mice containing the same number of repeats do not. He is interested in identifying cis acting sequences that mediate this instability. He has also started studying the SCA7 locus since in vivo it is quite unstable, while the androgen receptor locus is stable.

Yoshitaka Nagai has set up a screen to identify small peptides that protect against polyglutamine toxicity. He has screened a combinatorial library of peptides composed of 11 amino acids for molecules that interact with polyglutamine bait consisting of 62 glutamines and an amino-terminal GST. He has identified several peptides that bind with higher affinity to bait with a long polyglutamine tract (Q62) than to bait with a short polyglutamine tract (Q19). He co-expressed one of these peptides (called QBP1) in cell culture with Q81, and found that it localizes to aggregates. He is now looking to see if co-expression of QBP1 decreases toxicity.

Russ Margolis described his laboratory's work on SCA12. SCA12 has been studied in one large family with a disease characterized by tremor, progressing to cerebellar and other motor signs, and eventually to dementia. Cerebellar and cortical atrophy are present on MRI scans.

Margolis' laboratory used RED and 2-D RED to identify a CAG repeat expansion that segregates with disease in this family. They found a gene containing a polymorphic CAG tract (7 to 28 in unaffected family members and normal controls) that is expanded in patients with SCA12 to 65-75 repeats. The CAG repeat is at the 5' end of the gene encoding Pr55 beta, a regulatory subunit of protein phosphatase 2A that is specifically expressed in the brain. The CAG repeats are apparently within the promoter region of the gene, not the coding region; it is unclear whether they are present in the encoded mRNA. When the Pr55 beta promoter was coupled to a reporter gene, it was found that the repeat expansion was associated with higher levels of gene expression.

Josephine Dorsman described her *C. elegans* model of polyglutamine repeat disorders. She used two universal promoters to drive widespread expression, and observed neuronal loss and, in preliminary experiments, decreased life span. In addition, aggregates were not seen in animals using the hsp promoter after one heat shock. Leslie Thompson described her *Drosophila* model in which a polyglutamine repeat induces eye degeneration. If the polyglutamine is expressed in the context of a full-length protein that is normally expressed in the cytoplasm (disheveled), aggregation and nuclear localization as well as degeneration is prevented, consistent with a role for nuclear localization and protein processing. She is conducting a screen for suppressors that rescue the degenerative phenotype.

### **6. Summary**

Participants agreed on the need to develop further animal models of Huntington's Disease, to continue evaluation of the role of huntingtin as a modulator of transcription, and to further address the role of proteasome dysfunction in polyglutamine disease. An important issue to be addressed in the future will be how best to use array technology to address questions raised by these diseases. This, and other issues, will be discussed at upcoming HDF workshops.