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**Cover Page Footnote**
I would like to thank Dr. DebBurman for his help researching this project. I would also like to thank Julie Wang and Melissa Schramm for their help editing this paper. I would like to thank Quincy Roberts as well for his help in formatting the paper.

# The Hunt in Huntington: *What Causes Toxicity?*

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**Summary**

**Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG repeat mutation in exon 1 of the HD gene. This repeat expansion leads to an extended polyglutamine tract in the huntingtin (htt) protein. Symptoms of HD are associated with hyperkinectivity and include chorea as well as other motor abnormalities. Degeneration is most apparent in a region of the striatum called the caudate putamen. Loss of GABAergic spiny projection neurons in this area leads to motor problems. My lab has made significant contributions to the understanding of HD by developing the commonly used R6/2 transgenic mouse model. The development of this model has led to our discovery of htt protein aggregates in mice and humans, which are presumably associated with neurodegeneration and compromise of the degradation pathway. Evidence suggests that mutant htt alters gene transcription factors (CREB binding protein, and SP1), p53 dependent apoptosis, transport of BDNF, interactions with caspases and proteasomes, as well as mitochondrial function. Treatment strategies include environmental enrichment, anti-apoptosis drugs, and gene therapy. While a cure for HD remains to be uncovered, newly proposed mechanisms of toxicity should lead to promising clinical trials.**

**Introduction**

Huntington's disease (HD) is a neurodegenerative disorder that is characterized by the misfolding and subsequent aggregation of a particular protein as is the case in many other neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and prion diseases. HD affects approximately 5-10 people per 100,000, making it the most common inherited neurodegenerative disorder. The protein in question in HD is Huntingtin (htt). The misfolding of this protein is believed to be caused by a mutation in exon 1 of the *IT15* gene located on chromosome 4. The mutation is a trinucleotide repeat; this characterizes HD as a member of triplet diseases, which is caused by an excessive amount of repeats in a gene (1). One of the first identified trinucleotide repeat diseases is fragile x syndrome, caused by an abnormal amount of CGG repeats in a particular gene. The group of nucleotides that is expanded in HD is CAG. This sequence codes for the amino acid glutamine in the htt protein. An expansion of the CAG sequence in the HD gene leads to a longer stretch of glutamines, termed a polyglutamine (polyQ) region.

The number of CAG repeats determines toxicity. Individuals with 35 repeats or less do not develop the disease, whereas those with 36-39 have an increased risk of developing the disease. When the number of repeats is over 39 disease will develop, usually with symptoms presenting themselves between 35 and 50 years of age. An extreme form of HD also exists when the number of repeats is over 60, leading to juvenile onset of HD. Number of CAG repeats in the polyQ tract is inversely related with age of disease onset. There are several other polyQ disorders caused by mutations on different chromosomes, such as certain types of spinocerebellar ataxias (SCA), dentatorubral pallidoluysian atrophy (DRPLA), and spinal and bulbar muscular atrophy (SBMA). Like HD these follow autosomal dominant patterns of inheritance and are disorders characterized by problems with movement (2). Symptoms of HD have an emotional, cognitive, and motor component and are associated with hyperkinectivity. They can range from being barely noticeable to extremely severe. Chorea is a characteristic symptom and is defined as spontaneous, irregularly timed, randomly distributed, and abrupt movement (1). Other frequently observed motor abnormalities include sustained muscle contraction, rigidity, and tremor. The emotional disorder usually consists of depression and irritability, and the cognitive component compromises a subcortical dementia. The main region of the brain affected by HD is the striatum, specifically the caudate nucleus and putamun. Neurons lost in these regions are primarily γ-aminobutyric acid (GABA) releasing medial spiny projection neurons. Not only do neurons in this region show high levels of cell death, but protein aggregates termed intranuclear inclusions (NIIs) are also prevalent. Aggregates, believed to be a result of the incorrect folding of htt, while usually presenting themselves in the nucleus, can also be found in the cytoplasm of the cell (3).

The exact function of wild type htt is yet to be determined, but evidence suggests its involvement in vesicle transport, neuronal transport, endocytosis, and postsynaptic signaling. The protein is ubiquitously expressed and is localized in many subcellular compartments (2). The mutation occurring in the HD gene presumably leads to a gain of function in the mutant htt rather than a loss of function. This is based on the evidence that HD knockout mice die neonatally due to abnormal brain development. My lab and other colleagues have conducted studies showing that mutant htt may impair the transcription of specific genes found in htt aggregates, such as p53, CREB binding protein, and Sp1. Evidence for impairment of the ubiquitine-proteasome system (UPS) is also shown by the accumulation of chaperones, proteasomes, and ubiquitin in polyQ aggregates.

This paper will focus on the development of an animal model of HD which allowed for many of the aforementioned discoveries to be made. The importance of these findings in identifying the molecular mechanisms underlying the steps between polyQ expansions and aggregate toxicity will be discussed as well as current and future treatment options.

**A mouse model and discovery of aggregates**

In the mid to late 90's the main goal in my lab was to establish an efficient model of HD in order to better study the disease. After an initial attempt to construct an artificial chromosome in mutant yeast for injection into a murine model failed, a different approach was taken. We created several lines of mice that were transgenic for exon 1 of the human HD gene located on chromosome 4. By microinjecting this DNA fragment into mouse embryos, mice developed with a variety of different polyQ lengths. This

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fragment was sufficient to produce a progressive neurological phenotype that displays many HD characteristics. Four transgenic lines were established; one of these lines, the R6/2 line carrying 144 CAG repeats was the most successful and was established as a disease model (1).

Initially we were only able to detect several changes in R6/2 mouse brains that corresponded to human degeneration. Behavioral symptoms of HD present themselves at approximately 6 weeks of age. These include decreased motor function and a characteristic feet-clasping posture when suspended by their tails. R6/2 mice have a decreased size and body weight as well as decreased overall brain size. In a subsequent experiment we detected the presence of intranuclear inclusions (NIIs) in most brain regions; however, they were localized primarily in the cerebral cortex, cerebellum, and striatum (3). Extranuclear htt aggregates were observed as well. These are immunopositive for htt and are never found in control animals. In controls immunoreactivity for htt is in the cytoplasm. This suggests that wild type htt normally does not exist in the nucleus of cells. Aggregates can be observed as early as 3.5 weeks, showing that their formation precedes symptoms of neurodegeneration. Mice in all of the transgenic lines that exhibit symptoms have NIIs. This strongly implicates a causative role for NIIs in the generation of neurological dysfunction. I was very excited to discover that the presence of NIIs was shown in human postmortem tissue as well (4). While other studies reported change in neuronal membranes, such as the increase in nuclear indentation and the increase of nuclear pores, the presence of NIIs had not previously been detected. We were also able to show that R6/2 mice exhibit death of GABAergic neurons in the striatum, but this pathology does not present itself until approximately 13 weeks of age, the age around which R6/2 mice usually die. The fact that inclusion body formation precedes degeneration of the striatum also indicates that aggregate formation might induce cell death. Another finding that supports this notion is that NIIs are ubiquitinated. This suggests that mutant htt protein is targeted for proteolysis and degradation but is resistant to removal (5). After the identification of NIIs in our mouse model and human HD tissue, we sought to develop an in vitro model as well. Exon 1 of the human HD gene was used again to produce glutathione S-transferase (GST)-HD fusion proteins in E.*coli.* This resulted in the formation of high molecular weight protein aggregates. The filaments of these proteins have a ribbon-like morphology, which resembles both scrapie prions and β-amyloid fibrils in Alzheimer's disease. Additionally, these proteins are similar in appearance to aggregates detected by electron microscopy in R6/2 mice (5).

Loss of neurotransmitter receptors in the brain is a pathologic hallmark of patients with HD. Receptors affected are glutamate receptors (GluR), dopamine (DA), GABA, and muscarinic cholinergic receptors (mAchR). Several neurotransmitter receptors known to be affected by HD are also decreased in R6/2 mice. These include several types of GluRs, mAchRs, as well as DA receptors, but do not include GABA receptors. The loss of GluRs is a particularly interesting finding since loss of presynaptic GluRs might result in the unregulated release of glutamate. Increased glutamate release has been thought to play a role in the pathogenesis of HD. While findings support the similarity of R6/2 mice to human HD, they cannot be explained by generalized dysfunction of a specific striatal cell type (6,7,8).

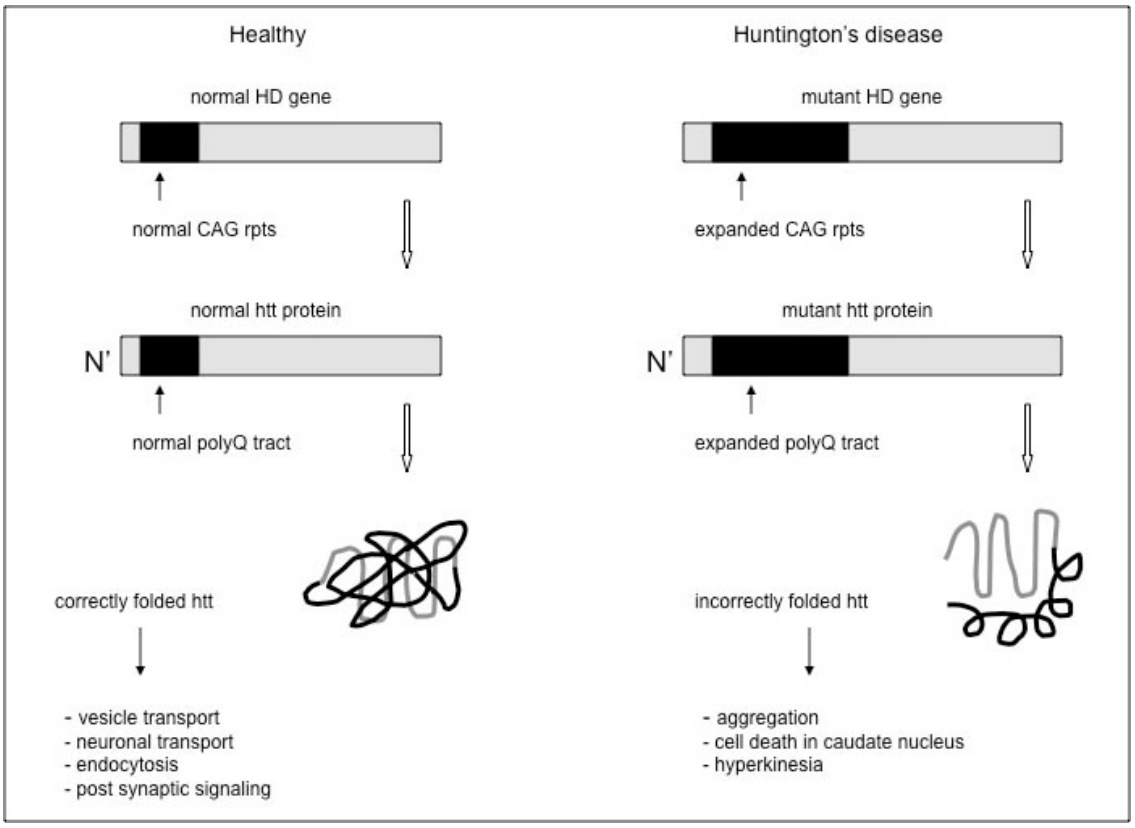


**Figure 1. Biological Basis for Huntington Disease:** An HD gene with expanded CAG repeats leads to mutant htt protein with an expanded polyQ region. This protein is unable to fold correctly. Consequent aggregation is associated with toxicity and neuronal death in the caudate putamen.

In order to confirm that mice of the R6/2 model suffered neurodegeneration in a way similar to humans, it is important to look at both characteristic pathological changes and changes in animal behavior indicative of motor impairment. As mentioned above, we observed that R6/2 mice clasp their feet towards their body when suspended from their tails whereas non transgenic mice will hang freely. There have been several models prior to the development of the R6/2 line that showed motor symptoms of HD including abnormalities, gait, and impairments in sensorimotor gaiting. A model of importance was, and still is, the 3-nitropropionic acid (3-NP) model. However, because this model and others like it induce disease neurochemically, associated changes in behavior are not truly progressive. The R6/2 model, however, does allow for this. Our model showed that symptoms of behavioral changes can be observed as early as 5-6 weeks of age in tests that involve swimming, balance and motor coordination on beams, and footprint tests. Symptoms become obvious at around 8 weeks of age when mice display stereotypical hind limb grooming movements and an irregular gait. We have shown that symptoms progress and performance on tests decreases as mice age. A test called the RotaRod test is an effective way of measuring motor impairment and is commonly used not only in HD experiments but also in other diseases involving motor abnormalities, such as Parkinson's disease and ataxias. The RotaRod test involves a rod suspended from the ground which can be set to different speeds, usually slightly higher than walking speed, on which mice have to walk. Mice are scored by how long they are able to remain on the rotating rod. Animals with severe motor impairments will not be able to remain on the rod for an extended amount of time. R6/2 mice exhibit decreased performance on the RotaRod as they age, confirming that our model indeed induces progressive neurodegeneration (9).

**DNA instability and changes in synaptic function in R6/2 model**

Once the R6/2 model of HD was established and we discovered the presence of htt aggregates, our lab and others were able to start using the model to study mechanisms by which neurodegeneration and aggregation occurs. The effectiveness of possible treatment options was also explored.

One of the first findings we came across while establishing the mouse model was that there is DNA instability in the highly expanded CAG repeat ranges. CNS structures in particular are shown to exhibit the most variability in CAG repeat number. This change in repeat length is called somatic repeat instability. Repeat instability is shown to increase as mice age (10). More recently, we were able to show evidence for the same repeat instability in human tissue and that it is most evident in the striatum, cortex, and brain stem. Most importantly, we showed that these changes can occur in terminally differentiated neurons. Development of instability has been linked to the DNA mismatch repair pathway. Different mismatch repair genes have different effects on CAG repeat expansion. MSH3 is shown to be important in maintaining instability. Cells in which MSH3 is absent are unable to change CAG repeat size in adulthood (11).

Knowing that substantial cell death does not occur in the R6/2 model until well after onset of symptoms, we set out to examine if other changes were occurring first. Human HD patients are also known to exhibit cognitive changes before classical symptoms of HD present themselves. Cognitive processes such as learning and memory are believed to be dependent on changes in synaptic function in certain brain regions, most importantly the hippocampus. We examined the baseline synaptic properties of the CA1 region of the hippocampus involved in cognition. Results suggested that normal synaptic transmission occurs in transgenic mice compared to control animals, but the mean amplitude of the action potential was significantly smaller in HD animals. Transgenic mice showed impairment in cognition when performing a cognition test called the Morris water maze. This indicates that altered synaptic plasticity may be contributing to the early cognitive deficit in presymptomatic HD patients (12).

**Transcription dysregulation in HD**

At this point we knew that NIIs were present in transgenic mice and HD patients, and we suspected their involvement in cell death. We were only able to prove this involvement after one important experiment showing that degeneration occurs in the same brain regions of transgenic mice and HD patients. More importantly, the study showed that degeneration occurs specifically in the anterior cingulated cortex, striatum, and cerebellum. A more focused study of aggregates showed a temporal progression of protein aggregation, inclusion formation, appearance of symptoms, and finally neurodegeneration (12).

In order for aggregation to take place several of the htt protein must undergo several changes. Normally, the htt protein is located in the nucleus. A portion of the N' terminal end of htt protein containing the polyQ expansion called httex1p is also localized to the nucleus, however, this is due to aberrant proteolytic cleavage of the protein. Htt interacts with the nuclear pore protein translocated promoter region (Tpr) which is involved in nuclear export. Mutant htt decreases the interaction with this protein and is therefore localized to the nucleus. Reducing expression of Tpr by RNA interference, or by deleting ten amino acids of N-terminal htt, which are essential for the interaction between htt and Tpr, increases the amount of htt in the nucleus (13). The consequences of nuclear localization of htt can be aggregation and errors in as well as suppression of gene transcription. Httexp1 is found to interact with the tumor suppressor protein p53 and CREB binding protein (CBP). Httexp1 prevents transcription of certain p53 activated promoters. CBP is a coactivator of several p53 regulated promoters. By direct sequestration of CBP to NIIs, mutant htt might repress transcription of these promoters. Changes in p53 expression can lead to changes in apoptosis since p53 plays an important role in regulating this form of cell death (14). One experiment of ours indicated that cell death by apoptosis is not playing a role in the R6/2 model of disease. It could be that altered p53 transcription leads to a decrease in apoptosis in HD (15). An important paper by another lab showed further evidence of p53 interaction with mutant htt. Mutant htt upregulates levels of p53 throughout brains of HD mice and patients. Genetic deletion of p53 suppresses neurodegeneration in flies and neurobehavioral abnormalities in mice (16).

A specific way that mutant htt interacts with the CREB-dependent transcriptional pathway is by inhibiting transcription of PGC-1α, a coactivator that regulates several metabolic processes including mitochondrial synthesis and respiration. Inhibition of PGC-1α is associated with mitochondrial dysfunction. Moreover, induced expression of PGC-1α reverses the effects of mutant htt in cultures striatal neurons (17).

There are several other known targets of htt-mediated transcriptional repression. Another lab showed that the gene specific activator protein Sp1 is a direct target of mutant htt. TAF4 is one of the TATA binding protein

associated factors in TFIID. TFIID is one in a series of transcription initiating factors and is recruited to the core promoter with the help of Sp1. Another transcription factor is TFIIF. Two of its subunits RAP30 and RAP74 bind RNA polymerase and help recruit this enzyme to TFIID. In a normal situation the RAP30 interacts with RAP74 in order to function correctly. However, parts of hhtexp1 of mutant htt interact with the region of RAP30 that normally interacts with RAP74. Normal htt is shown to not interact with RAP30. The presence of free RAP74 is speculated to mediate toxicity and mechanisms that inhibit the transcription of certain genes. Overexpression of RAP74 leads to increased cell death in cultured mouse striatal cells whereas overexpression of RAP30 decreases cell death in the same type of culture (18).

Another target of altered gene transcription is the cholesterol biosynthetic pathway. The transcription of genes in the cholesterol synthetic pathway is regulated via the activity of sterol regulatory element-binding proteins (SREBP's). Cholesterol is synthesized in the brain and makes up a major component of myelin. It is also crucial for optimal neurotransmitter release, and a depletion of cholesterol can lead to a gradual loss of synapses. Reduced cholesterol levels have been shown to play a role in Alzheimer's disease as well as Parkinson's disease, and in an interesting experiment my lab showed up to a 50% decrease in the amount of SREBP's in HD human and mouse brains. We showed that treatment of striatal neurons expressing mutant htt with exogenous cholesterol reduces cell death (19). This presents an interesting treatment option for HD.

**Impairment of the UPS system**

Another aspect of normal cellular function that is shown to be compromised in HD is the ubiquitin proteasome system (UPS). Proteasomes' important function in the cell is to degrade damaged or incorrectly folded proteins. Proteins are tagged for degradation by a molecule called ubiquitin. Reduced function of the UPS system can lead to the presence of incorrectly folded proteins as is the case in HD. One molecule that my lab has shown to inhibit proteasome activity is arfaptin 2. Expression of arfaptin 2 is shown to be upregulated in sites of neurodegeneration. Arfaptin not only localizes to aggregates in HD mouse brains, it is also involved in regulating the aggregation of mutant htt. Increased expression of arfaptin results in increased aggregation by affecting the proteasome complex. Aggregates may in turn further harm the UPS system (20).

Many other factors that may harm the UPS system have been discovered as well. For example, just last year we showed several changes in the ubiquitin system in cultured HD cells. Modification of proteins with polyubiquitin chains regulates important cellular processes, so it makes sense that altered ubiquitin signaling can have many effects on neuronal function and survival. We showed that the lys48 linked polyubiquitin chain accumulates early in pathogenesis in R6/2 as well as human HD brains. Chains lys63 and lys11, which are not usually associated with proteosomal targeting, also accumulate in the R6/2 brain (21). SUMOylation is also shown to interact with httexp1. SUMO molecules can compete with ubiquitin to bind to httexp1. In this case the protein will not be marked for degradation as it would if tagged with ubiquitin. SUMOylation of httexp1 is thereby shown to promote neurodegeneration (22). Evidence against impairment of the UPS system has also been shown in my lab. We recently proposed that the proteasome activator REGγ could contribute to UPS impairment by inhibiting the cleavage of glutamine-glutamine bonds. When we genetically reduced REGγ levels in R6/2 mice, however, it did not change the well defined neurological phenotype or affect levels of NIIs (23).

Two more important mechanisms involved in the progression of HD have been found. Caspase activity is one of them. As mentioned earlier, certain proteases cleave htt within the N-terminal region and this and expanded N-terminal fragments lead to toxicity. Htt is cleaved by caspace 3 and 6 in vitro, and fragments cleaved by these caspases are detectable in HD brains. Graham and colleagues showed in 2006 that cleavage of caspace 6, but not of caspace 3, is important in mediating neurodegeneration. Inhibition of caspace 6 in vivo was shown to significantly inhibit neurodegeneration (24).

**Chaperone proteins and changes in the cytoplasm**

A decrease in levels of chaperone proteins is another change that specifically leads to protein misfolding. Once information for proteins has been translated it is crucial that proteins fold correctly. Chaperone proteins help with such folding and also with the rescue of previously aggregated proteins. The manipulation of chaperone levels has been shown to inhibit aggregation and rescue cell death in several models of disease. For example, two chaperone proteins Hsp40 and Hsp70 have been shown to inhibit the aggregation of htt proteins with expanded polyQ tracts (25). My lab showed that the specific decrease of brain levels of chaperones Hdj1, Hdj2, Hsp70, αSGT, and βSGT contributes to disease pathogenesis and are therefore of importance in either the correction or prevention of mutant htt misfolding (26).

Besides intranuclear aggregates, cytoplasmic degeneration can occur as well. My lab generated a line of mice in which nuclear localization or nuclear export signals were placed N-terminal to httexp1. Cytoplasmic degeneration was still shown to occur when a nuclear localization signal was present (27). Expression of a cytoplasmic polyQ repeat protein leads to axonal blockages in flies resulting in subsequent deficits in axonal transport. Nuclear htt causes cytoplasmic aggregation but not axonal blockages. This suggests that htt acts by two exclusive mechanisms, one that causes nuclear and cytoplasmic aggregation and another that leads to deficits in axonal transport (28).

Deficits in axonal transport can lead to changes in transport of neurotransmitters (29). One example of a neurotransmitter whose transport is affected is BDNF. BDNF is produced in the striatum and is transported to the striatum. While deficits in BDNF are often associated with reduced BDNF transcription, another lab shows that BDNF transport is reduced by interaction with Huntingtin-associated protein 1 (HAP1), which is important in regulating the microtubule-based motor complex. By mutant htt interaction with HAP1, the motor machinery of mictrotubules is compromised which leads to reduced BDNF transport and subsequent neurotoxicity (30**).** Some others argue that full length htt can suppress neurodegenerative phenotypes and increase synaptic transmission of neurotransmitters (31).

**Possibilities for treatment and the role of the R6/2 model in development thereof**

There is currently no known cure for HD; the only drugs available are those that aim to alleviate symptoms. My lab and others have shown the importance of environmental enrichment in the R6/2 model. Transgenic animals that were in an enriched environment with a larger, warmer cage with different types of bedding, more toys, and more possibilities to exercise showed increased RotaRod performance as well as an increase in brain weight compared to animals in a

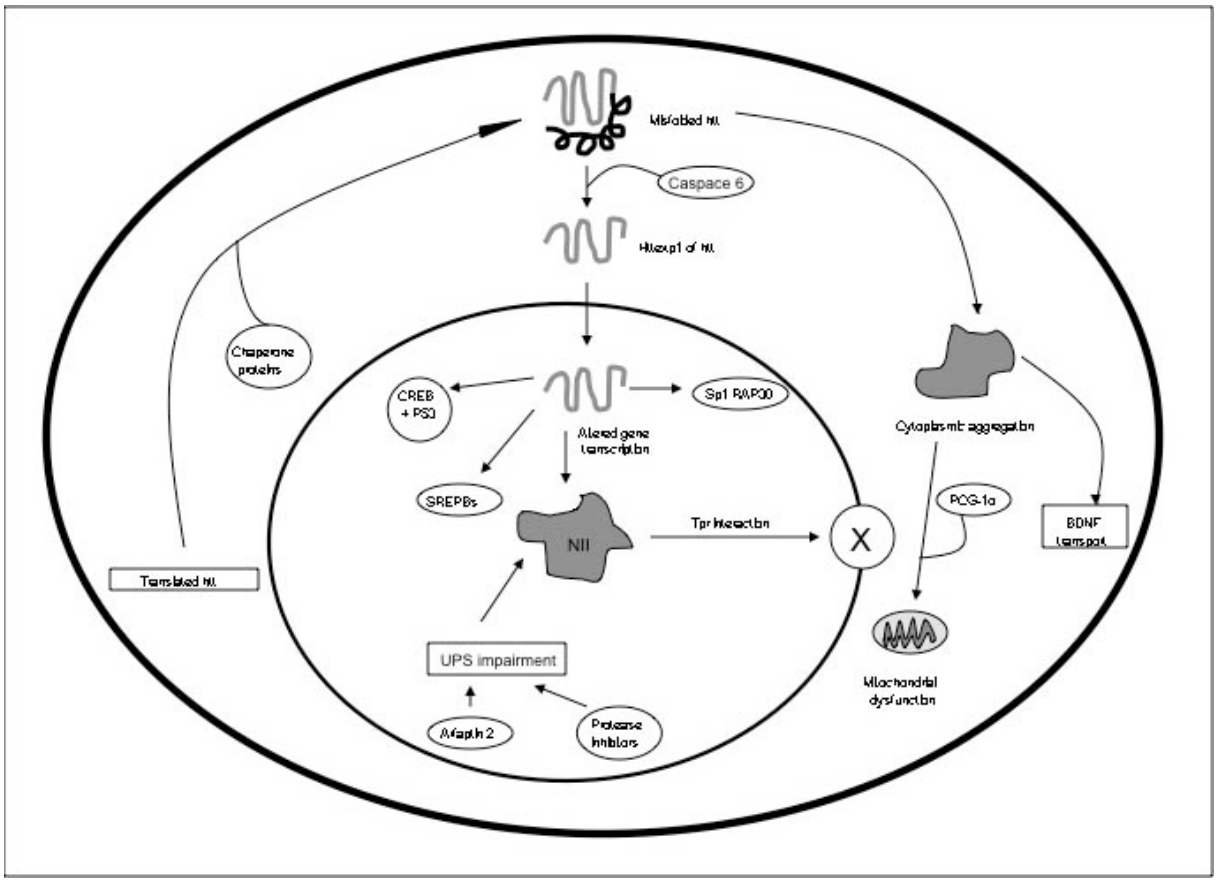


**Figure 2.** A summary of well characterized action of mutant htt incuding transcriptional disregulation, UPS impairment, as well as intranuclear and cyroplamsic aggregation.

normal, non-enriched cage (32,33). This has implications for human HD patients. An environment that is stimulating, particularly in academic and occupational attainment early on in life as well as throughout, may prevent a variety of diseases or delay their onset.

Because caspase 6 is involved in cleavage of httexp1 of mutant htt, caspase 6 inhibitors are currently being explored as treatment options. My lab has also sought for molecules that inhibit polyQ aggregation. We found one molecule called C2-8 that was shown to inhibit aggregation in cell culture as well as a *drosophila* model of HD (34). Further investigation into the properties of this molecule is ongoing. It seems as though a combination of therapeutics would be the best approach to reduce symptoms of HD. From evidence brought forward in this paper we can propose that cholesterol treatment, expression of PCG-1α, deletion of p53, and overexpression of certain chaperone proteins could be beneficial in treatment of HD. These treatments, however, could have possible negative effects as well, especially deletion of p53 which is often seen as the "guardian angel" of the cell.

It is also important to explore different target treatments other than those that inhibit aggregation. Some researchers have found evidence that formation on NIIs might actually promote cell survival by grouping misfolded proteins together rather than having them diffuse throughout the cell (35). It is important to be extremely careful and critical when researching treatment options. This involves proper use and understanding of the R6/2 model. For example, the widely used tetracycline antibiotics, were explored as treatment options since they are inhibitors of certain caspases. Two closely related compounds minocycline and doxycycline were examined because they are able to cross the blood brain barrier. In one experiment minocycline was shown to be beneficial in the R6/2 model. There were no effects on aggregation, but improvement in RotaRod performance was observed. I want to stress the fact that while behavioral evidence is useful to support pathological evidence of improvement it is not to be used alone. An experiment with no reduction in cell death or aggregation cannot be said to be a success. We tested effects of minocycline and doxycycline ourselves but did not observe any pathological improvement either (36). It is also important to realize that the R6/2 model only contains exon 1 of the human HD gene. If one wants to examine the effects of full length htt, HD knock in models will need to be used. Also, the R6/2 model is an accelerated model of disease which induces HD early on in the life of a mouse. The amount of 144 CAG repeats which R6/2 mice have is enough to induce juvenile HD in humans. Because this is an extreme situation, the accelerated phenotype of this model might not be able to detect subtle improvements due to therapy (37). Having said this, the R6/2 model can continue to be beneficial in the discovery of HD mechanisms and the search for therapeutic strategies.

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