The Molecular Pathology of Huntington’s Disease (HD)

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Abstract: Huntington’s disease (HD) is one of nine known neurodegenerative conditions caused by CAG trinucleotide repeat expansions that are translated into abnormally long polyglutamine tracts in the mutant protein. This review describes the clinical and pathological features of HD and considers the genetic and transgenic/knockout mouse data supporting gain-of-function vs. loss-of-function mechanisms whereby the mutation may cause disease. Intranuclear aggregates (also known as inclusions) are one of the pathological hallmarks of all of the polyglutamine expansion diseases. A major focus of the review is a detailed consideration of the debate as to whether aggregates/aggregation are pathogenic, deleterious or epiphenomena, drawing on data from cell-based and animal models of different polyglutamine diseases and also from the polyalanine codon reiteration disease, oculopharyngeal muscular dystrophy, which manifests intranuclear nuclear inclusions. I will describe how cells deal with aggregate-prone and misfolded proteins using chaperones and various degradation pathways. Using data from animal and cell-based models, the review considers some of the different but non-mutually exclusive mechanisms whereby the HD mutation may cause disease, including early changes in gene transcription, production of reactive oxygen species, aberrant protein-protein interactions and abnormal cellular susceptibility to glutamate. Understanding possible pathogenic mechanisms for HD has provided a rational basis for intervention strategies, ranging from antibodies/peptides that prevent mutant protein aggregation to drugs that enhance mitochondrial function and protect against excitotoxicity.

SYMPTOMATOLOGY OF HD

HD is an autosomal dominant neurodegenerative disease associated with a triad of symptoms, including movement disorders, cognitive deterioration and psychiatric disturbances. Symptoms of the disease begin insidiously, most commonly between the ages of 35 to 50, but the age of onset can vary from early childhood until old age. The disease is relentlessly progressive and fatal 15–20 years after the onset of symptoms. Disturbances of motor function are classical features of the disease. They include choreiform involuntary movements of the proximal and distal muscles, and progressive impairment of co-ordination of voluntary movements [1-3]. In patients with juvenile-onset HD, the signs and symptoms are somewhat different; they include bradykinesia, rigidity and dystonia, and chorea may be completely absent. Involuntary movements may take the form of tremor, and affected children often develop epileptic seizures [4].

The cognitive difficulties usually start with a slowing of intellectual processes. In contrast to Alzheimer’s disease, patients with HD have problems with retrieval of established memory rather than formation of new memories. Cognitive losses accumulate progressively and late-stage patients with HD have profound dementia. Emotional disturbances in the form of depression and manic-depressive behaviour are common features. Personality changes, including irritability, apathy and sexual disturbances often accompany the psychiatric syndrome [5,6]. The rate of suicide has been estimated at 5-10% [7,8].

NEUROPATHOLOGY

HD is characterised by a striking specificity of neuronal loss. The regions most affected are the striatum, where there is typically 50-60% loss of cross-sectional area from the caudate nucleus and the putamen in advanced disease. In the striatum, the most sensitive cell population are the medium spiny neurons, which show denticritic changes including recurving of denticles and altered density, shape and size of spines. Among spiny neurons, enkephalin-containing neurons projecting to the external globus pallidus externa are more susceptible compared to substance-P-containing neurons projecting to the internal globus pallidus [9,10]. Although the pathology of HD is most obvious in the striatum, it is likely that much of the degeneration occurs elsewhere - the average brain of someone who has died of HD weighs about 300 g less than normal and the striatal atrophy can only account for about 50g of this loss [11].

There is loss of cortical volume, which affects predominantly the large pyramidal neurons in layers III, V and VI. While this loss is overt in advanced cases, it may be one of the earlier changes in the disease [12]. In advanced cases, there is also loss of neurons in the thalamus, substantia nigra pars reticulata and in the subthalamic nucleus. There is also loss of volume in the globus pallidus, which is primarily due to loss of striatal fibre connections, rather than to loss of neurons. Cerebellar atrophy is most frequently reported in cases with juvenile onset disease. Fibrillary astrogliosis mirrors the loss of neurons as the disease progresses (reviewed in [9,10]).
Vonsattel and colleagues described a grading system for standardisation of HD pathology based on gross and microscopical examinations [9]. It is interesting that the mildest category, grade 0, is grossly indistinguishable from normal brains but cell counting can show up to 40% loss of neurons in the head of the caudate nucleus in such brains.

**GENETICS OF HD**

The HD gene and mutation were identified in 1993 [13]. The gene is located on the short arm of chromosome 4 (4p16.3) and encodes a large protein called huntingtin that contains more than 3000 residues. Exon 1 of the wild-type gene contains a stretch of uninterrupted CAG trinucleotide repeats, which is translated into a series of consecutive glutamine residues, a polyglutamine (polyQ) tract. Asymptomatic individuals have 35 or fewer CAG repeats and HD is caused by expansions of 36 or more repeats [14]. There is an inverse relationship between CAG repeat number and the age of onset of symptoms: the greater the number of CAG repeats, the earlier the age of onset [15]. Most adult onset cases have 40-50 CAG repeats, whereas expansions of >55 repeats frequently cause juvenile-onset disease. Incomplete penetrance has been observed in individuals with 36-39 repeats - some individuals in their 9th and 10th decades with alleles in this size range have no signs, symptoms or gross neuropathological features of HD [14,16]. About 70% of the variance in the age at onset of HD can be accounted for by CAG repeat number. Family studies suggest that a component of the residual variance not associated with the CAG repeat number, may be accounted for by additional genetic factors [17]. One possible candidate modifying gene is the GluR6 kainate receptor [18] – the effect that we reported of genotypes at this locus on age-at-onset of HD, after accounting for the CAG repeat length, has been replicated by MacDonald et al. [19].

HD has been considered to be a true dominant disease where the severity is similar in patients with one or two mutant alleles. However, this interpretation is based on data from only a few homozygote cases (reviewed in [20]). A recent study on the largest cohort of homozygotes assessed to date suggests that while the age at onset is similar between homozygotes and heterozygotes matched for the larger repeat size, the progression of disease may be more rapid in homozygotes [20]. One interpretation of these data is that CAG repeat length is a more important factor than gene dosage. However, since cleavage of the full-length protein to an N-terminal toxic fragment may be a key step in the pathogenic pathway (see later), another possibility is that the processing events leading to the formation of this toxic fragment or fragments is rate-limiting.

HD is a member of a growing group of diseases caused by CAG repeat/polyglutamine expansions that include spinocerebellar ataxias types 1, 2, 3, 6, 7 and 17, spinobulbar muscular atrophy (SBMA) and dentatorubral-pallidoluysian atrophy (DRPLA) (reviewed in [21]).

HD is an autosomal dominant condition – one mutated gene is sufficient to cause the disease, in spite of the presence of a normal gene inherited from the other parent. Genetic and transgenic data suggest that the mutation confers a novel deleterious function on the protein. In humans, hemizygous loss of one of the two huntingtin genes has been observed as a result of either a terminal deletion of one chromosome 4 (which includes the HD gene) in patients with Wolf-Hirschhorn syndrome [22], or a balanced translocation with a breakpoint between exons 40 and 41, physically disrupting the HD gene in one female patient [23]. However, this hemizygous inactivation of huntingtin does not cause an HD phenotype. In addition, mice that have only one functioning HD gene do not show features of the disease [24-27].

A gain-of-function is also supported by observations in the related polyQ disease spinobulbar muscular atrophy (SBMA), which is caused by a CAG repeat expansion in the androgen receptor. Males with SBMA develop progressive weakness and muscle atrophy due to loss of their motor nerve supply and mild androgen insensitivity [28]. This contrasts with the effects of point mutations elsewhere in the gene, which can result in severe androgen insensitivity but never cause a neuromuscular disease. Indeed, the neuromuscular features of SBMA are not even seen in a patient with a complete androgen receptor gene deletion.

This gain-of-function model was elegantly demonstrated in a mouse model, where 146 CAG repeat sequence was inserted into the hypoxanthine phosphoribosyltransferase (HPRT) gene, which is not involved in any CAG-repeat disorders. While previous work had shown that inactivation of the HPRT gene in mice does not cause deleterious effects, these mutant mice produced a polyglutamine-expanded form of the HPRT protein and developed a late-onset neurological phenotype that progressed to premature death [29].

Wild-type huntingtin is widely expressed and is found mainly in the cytoplasm, where, in neurons, it is associated with vesicle membranes and microtubules. Huntingtin appears to be associated with clathrin via the huntingtin interacting protein Hip-1 [30-34]. Wild-type huntingtin is necessary for development, as homozygous knockout mice show embryonic lethality [24-26]. This may be related to observations that overexpression of wild-type huntingtin protects cells against a variety of apoptotic stimuli [35], probably by inhibiting pro-caspase-9 processing [36]. Wild-type huntingtin can protect against apoptosis in the testis of mice expressing full-length huntingtin transgenes with expanded CAG repeats [37] and overexpression of wild-type huntingtin significantly reduced the cellular toxicity of mutant HD exon 1 fragments in both neuronal and non-neuronal cell lines [38]. This suggests that overexpression of wild-type huntingtin can be protective in different cell types and that it can act against the toxicity caused by mutant huntingtin.

In addition to protecting against polyglutamine expansion toxicity, wild-type huntingtin may also play a role in HD by upregulating the transcription of brain-derived neurotrophic factor (BDNF) [39]. This activity appears to be reduced in the disease state and Zuccato and colleagues [39] have proposed that this may contribute to HD pathology by reducing neurotrophic support from cortical neurons to striatal neurons.

Gervais et al. have proposed that the polyglutamine expansion in huntingtin may cause disease as it reduces
binding of the mutant protein to Hip-1 [40]. Free Hip-1 binds to Hippi (Hip-1 protein interactor) forming pro-apoptotic Hip-Hip-1 heterodimers. This heterodimer can recruit procaspase-8 into a complex of Hippi, Hip-1 and procaspase-8, and launch apoptosis through components of the ‘extrinsic’ cell-death pathway. Gervais et al. argued that the polyglutamine expansion in mutant huntingtin liberates Hip-1 so that it can form a caspase-8 recruitment complex with Hippi [40]. This process is unlikely to be the main or only mechanism for disease - HD-like pathology is seen in transgenic mouse models expressing both full-length and N-terminal mutant huntingtin constructs in mice with two endogenous huntingtin genes and while mutant huntingtin does not bind Hip-1 as well as the wild-type protein it does still bind Hip-1 [41]. Therefore, these transgenic mice are expected to have more Hip-1 bound to huntingtin, compared to wild-type mice.

POSSIBLE DISEASE MECHANISMS

A wide range of different mechanisms have been proposed to explain how the HD mutation causes cell dysfunction and death. I will discuss selected processes that I believe to be important. There are possible connections between many of the processes discussed, based on knowledge from other systems (e.g. reactive oxygen species and apoptosis, energy production and ubiquitin-proteasome function etc.). However, since the roles of many of the proposed mechanisms as primary factors in pathogenesis in HD patient brains have not been conclusively demonstrated, I believe that it would be unwise and possibly misleading to speculate on too many connections between putative pathogenic pathways. It is possible and indeed likely that a number of processes are acting in concert in HD as the mutation results in a chronic insult acting over decades. Furthermore, there may some pathways that are shared between the different polyglutamine diseases and even between different diseases associated with abnormal intracellular protein aggregation.

HUNTINGTIN AGGREGATES

In 1996, Bates and co-workers created a transgenic mouse which expressed exon 1 of the human HD gene containing different numbers of CAG repeats, under the control of the human huntingtin promoter [42]. Mice expressing 18 CAG repeats developed normally and remained healthy. By contrast, mice that expressed 113-156 CAG repeats developed progressive neurological symptoms together with some other clinical features similar to HD. These mice developed intraneuronal aggregates (inclusions) in nuclei and in neuronal processes [43]. Similar aggregates were identified in post mortem human HD brains in cortical and striatal neurons [44] and in dystrophic neurites [45], but not in the globus pallidus or cerebellum [46]. In HD brains these inclusions comprise truncated derivatives of the mutant proteins, which only appear to be recognised by antibodies to epitopes close to the expanded polyglutamines. The inclusions are spherical, ovoid or elliptical in shape and are concentrated in neurons in areas of the brain which degenerate in HD. Electron microscopy revealed that the inclusions were heterogeneous in composition and contained a mixture of granules, straight and tortuous filaments and

many parallel and randomly oriented fibrils. Intraneuronal aggregates are a pathological common denominator for polyglutamine diseases, since similar structures are seen in brains from cases with all of these diseases. Inclusions in juvenile onset cases occur predominantly in the nucleus, while a greater proportion of aggregates in adult-onset cases are cytosolic [46]. This may be a factor accounting for some of the differences in presentation between adult-onset and juvenile HD.

A causal role for these inclusions in polyglutamine disease pathology has been suggested, as they appear before the signs of disease in a transgenic mouse expressing exon 1 of the HD gene with expanded repeats [43]. In addition, the numbers of inclusions in the cortex of HD patients correlates with CAG repeat number [47]. Inclusion formation in vivo in cultured cells also correlates with susceptibility to cell death [48-52]. In striatal cultures, neuritic aggregates block protein transport in neurites, and cause neuritic degeneration before nuclear DNA fragmentation occurs [53]. These authors suggested that the early neuropathology of HD may originate from axonal dysfunction and degeneration associated with huntingtin aggregates. Reduction of polyglutamine inclusion formation, by overexpression of heat shock proteins and bacterial and yeast chaperones (that are unlikely to directly affect cell death pathways), is also associated with decreased cell death in vitro [54-56] (Fig. 1A).

[Diagram]

Fig. (1A). Formal possibilities of the relationship of aggregation to cell dysfunction/death in HD.

On the left, aggregation leads to death; in the middle, aggregation is an epiphenomenon unconnected to dysfunction/death; on the right, death/dysfunction causes aggregation. Experiments in cell culture and in vivo support the model on the left – chaperones and antibodies that reduce aggregation but do not affect cell death pathways reduce cell death [54-58,80]. On the other hand, reduction of cell death (e.g. with caspase inhibitors) does not reduce aggregation of HD exon 1 constructs in cell culture [166]. The central model is unlikely, since it predicts that chaperones would reduce aggregation but increase cell death.

A number of recent studies strengthen the claim for aggregates playing a role in disease. In the first, Koshnan et
al. studied a panel of monoclonal antibodies that they developed to various epitopes on huntingtin [57]. In cell culture models they showed that the antibody that significantly inhibited aggregation also inhibited cell death. In contrast, the two antibodies that stimulated huntingtin aggregation increased cell death. Kazantsev and coworkers have developed a related approach by designing suppressor polypeptides that bind mutant Htt and interfere with the process of aggregation in cell culture [58]. In a Drosophila model, the most potent suppressor inhibits both adult lethality and photoreceptor neuron degeneration. Furthermore, this work showed a strong correlation between the development of aggregates and cell death in the Drosophila model [58].

Wetzel and colleagues provided further support for a role of aggregates in experiments where they synthesized polyQ peptides in vitro and introduced these into cells in culture [59]. The addition of a nuclear localization signal to the peptides resulted in nuclear aggregate formation and cell death [59]. A pathological role for aggregation is also consistent with the observation that the predicted lag times for polyQ aggregation based on in vitro data correlate with the age-at-onset/CAG repeat curves seen in HD [60,61].

The failure to always see a correlation between inclusion formation and cell death/pathology in HD models has led some to question the role of aggregates. Saudou et al. observed a dissociation between inclusion formation and cell death in primary striatal neurons after inhibition of ubiquitination [62]. They tested the effect of inhibiting ubiquitination on inclusion formation and cell viability. The inclusions seen in patients' brains and in in vivo and in vitro models of polyglutamine diseases are ubiquitinated. This process is used by cells to tag misfolded proteins and target them for degradation. Saudou et al. showed that expression of a dominant-negative ubiquitin-conjugating enzyme mutant reduced the proportion of cells with aggregates but increased cell death caused by huntingtin constructs containing expanded repeats, in the cells remaining on the dishes after 6 days [62]. However, inhibition of ubiquitination also resulted in increased cell death in cells expressing “wild-type” huntingtin constructs with 17 repeats. It is not clear if the results were simply due to an additive effect of the polyglutamine insult and the defective ubiquitination, resulting in earlier death of cells expressing mutant huntingtin with smaller inclusion loads. This scenario would result in fewer adherent cells with visible inclusions after the 6 day experiment – dead cells do not remain attached to culture dishes for long.

Cummings and colleagues showed that a mutation in the E6-AP ubiquitin ligase reduced nuclear inclusion frequency but accelerated polyglutamine-induced pathology in SCA1 mice [63]. While these data suggest that large visible inclusions may not be required for cell death, the authors consider other possibilities which are consistent with a pathological role for inclusions. For instance, ubiquitination of ataxin-1 may not be E6-AP-dependent. The deletion of this enzyme may affect the turnover of other proteins, which at abnormally high steady-state levels may enhance the cellular sensitivity to the SCA1 mutation (or aggregates) [63].

Klement et al. suggested that aggregate formation may not be a prerequisite for pathology, since similar Purkinje cell pathology and ataxic phenotypes were observed in mice expressing the SCA1 gene with 77 repeats, with or without a deletion of the ataxin-1 self-association domain [64]. The mice expressing transgenes without the self-association domain had no intranuclear inclusions in their Purkinje cells, in contrast to the mice expressing the entire mutant gene. This conclusion may be simplistic, since later reports suggest that the phenotype in the mice with the deleted self-association domain progressed significantly more slowly than in mice with the full-length mutant gene [65]. Thus, inclusion formation may affect disease progression. These data need to be interpreted carefully, since no data were presented for mice with normal repeat lengths containing deletions of the self-association domain in SCA1 and Perutz suggested that removal of the self-association domain from the normal SCA1 gene may itself cause abnormal protein folding and a SCA-like phenotype [66].

Lansbury and colleagues have suggested that the aggregation process itself may be toxic, possibly with the most toxic species being oligomers/microaggregates, rather than aggregates that are easily visible using light microscopy [67]. Microaggregates have been identified by electron microscopy in the YAC transgenic mouse model made by Hayden’s lab [68]. They can be around 50nm in diameter [68]. Such microaggregates may be very difficult to detect or exclude in humans or animal models. A typical cortical neuron is about 25 um diameter and EM sections are usually 50 nm thick – 500 sections per neuron. Thus, if there were one 50nm microaggregate per cell (or five microaggregates in 20% of cells), one would only see such an aggregate in about 1/500 EM cell/sections. Thus, the failure to observe such microaggregates using EM does not exclude their existence even in the majority of neurons. Recent biochemical data from Ross and colleagues have confirmed that mutant huntingtin fragments form protofibril and globular intermediates that eventually become aggregates [69].

Further work needs to be done in order to clarify the controversy regarding the pathogenic role of aggregates. This may have a much wider relevance, since the phenomenon of ubiquitinated inclusion bodies is not confined to polyglutamine diseases. Indeed, it seems to be an emerging theme in many other late-onset neurodegenerative diseases like Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis and prion diseases [70]. Recently, another class of codon reiteration disease associated with β-sheet aggregates has been discovered, exemplified by autosomal dominant oculopharyngeal muscular dystrophy (OPMD) [71]. OPMD usually presents in the sixth decade with progressive swallowing difficulties, ptosis and proximal limb weakness. Its pathological hallmark is the presence of nuclear filamentous inclusions in skeletal muscles. OPMD is caused by the abnormal expansion of a (GGC)k trinucleotide repeat in the coding region of the poly(A) binding protein 2 gene (PABP2); a (GGC)k repeat is expanded to (GGC)k,13 in patients [71]. In PABP2, (GGC)k codes for the first 6 alanines in a homopolymeric stretch of ten alanines. Thus, disease is associated with expansions of 12 or more uninterrupted alanines in this exclusively nuclear protein. A number of other autosomal dominant diseases,
including synpolydactyly and a form of cleidocranial dysplasia, are also caused by polyalanine expansions in nuclear proteins (the transcription factors HOXD13 and CBFA1) [72,73].

A gain-of-function mechanism for polyalanine expansion mutations in OPMD would be consistent with experiments from our lab [74] and from Rouleau’s group [75]: cells overexpressing long alanine repeats, either in the context of PABP2 (17 alanines - A17) or fused to green fluorescent protein (GFP) (A19,37), show aggregate formation and increased cell death, compared to wild-type constructs which did not aggregate (PABP2 - A106, GFP - A17). PABP2 regulates the elongation of poly(A) tails and polyadenylation of mRNAs and this function is not impaired in OPMD myoblasts [76]. Further evidence against a loss of function mechanism in OPMD comes from studies of the Drosophila PABP2 orthologue [77], which lacks the first 54 residues of the mammalian proteins [71,78]. Despite lacking the domain that is mutated in OPMD (starting at residue 2 of human PABP2), the Drosophila protein stimulates poly(A) polymerase and is able to control poly(A) length in reconstituted mammalian polyadenation systems [77].

A causal role for aggregation in the cell death in tissue culture models of OPMD is supported by complementary data from our lab and Rouleau’s group. Rouleau and colleagues found that oligomerization of PABP2 is mediated by two potential oligomerization domains (ODs) – deletions in either one of these domains inactivated oligomerization of mutant PABP2 and also reduced the cell death caused by this protein [79]. In our experiments, we showed that a number of different chaperones, including bacterial and yeast derived constructs reduced the aggregate formation and cell death caused by mutant PABP2. Since two of the chaperones that were protective could not protect against the pro-apoptotic effects of H2O2 or staurosporine, it is likely that the protection against cell death was related to their effects on reducing PABP2 aggregation or misfolding [80]. While the mechanisms, structures and contents of polyA aggregates may differ from polyQ aggregates to some extent, it is tempting to speculate that the process of intracellular protein aggregation may perturb some similar processes. I believe that this is an important hypothesis to consider and test, given the large number of different diseases associated with this phenotype.

While the effects of chaperones on aggregation and cell death strongly support a correlation between the appearance of aggregates and cell death/dysfunction, they may be compatible with another theoretical model where aggregation would not necessarily be causally related to cell death. The mutant monomeric proteins may exist in two monomer conformations. Here the mutant protein can exist in two monomer conformations. One conformation is benign, the other is toxic and would be aggregate-prone. In this model, chaperones may prevent the conversion of the benign to the toxic monomeric conformation. However, the data on Congo Red suggest that it prevents the conversion of protofibrils to mature fibrils and therefore acts in the aggregation process [69]. Since Congo Red is protective in cell models and in vivo, this argues for a deleterious role for aggregation in disease pathogenesis [81].

Recent data suggest that interruption of aggregation may indeed be beneficial. Congo red blocks the conversion of mutant huntingtin protofibrils into mature fibrils [69] and Sanchez et al. showed that Congo red reduced aggregation and cell death in HD cell models [81]. The latter study also reported that infusion of Congo red into an HD mouse model by the intraperitoneal and intracerebroventricular routes improved survival, weight loss and motor function, compared to untreated mutant mice [81]. These data provide important insights into the role of aggregation of polyglutamine disease pathology and suggested that the beneficial effects of Congo red were due to its anti-aggregate properties. Unfortunately, the therapeutic potential for Congo red in HD may be minimal due to its very poor blood-brain barrier permeability [82].

**THE ROLE OF UBIQUITIN, PROTEASOMES AND CHAPERONES**

Cells have a complex array of chaperone proteins that assist in the folding of normal proteins, and recognition and handling of abnormally-folded or aggregate-prone proteins. Several of the heat shock proteins (HSPs) are involved in modulation of protein folding pathways, promoting proper protein assembly in an ATP-dependent manner and preventing misfolding and aggregation under both normal and stress conditions [83]. HSP70 and HSP40 family members are associated with huntingtin exon 1 aggregates in cell models [52,84,85]. Overexpression of the human HSP40 homologues HDJ-1 and HDJ-2 and/or HSP70 family members in vitro reduced aggregation of ataxin-1, ataxin-3 and the androgen receptor with expanded polyglutamine
proteins in cell models [54,55,86]. However, overexpression of HDJ-2 did not alter the aggregation of exon 1 of huntingtin containing 72 repeats in two neuronal-precursor cell lines and actually increased aggregation in COS-7 cells [52]. Thus, overexpression of this HSP may increase or decrease aggregates in different cell types or with different polyglutamine containing proteins.

An important intracellular pathway for reducing the levels of misfolded proteins is the ubiquitin-proteasome pathway. Intranuclear inclusions seen in patients’ brains and in in vitro models of polyglutamine diseases are ubiquitinated and sequester components of the proteasome [52,54,87]. Proteasome inhibitors increase steady-state levels of such proteins [88] consistent with the hypothesis that the proteasome degrades proteins containing expanded polyglutamine tracts. Another major route for degradation of proteins with expanded polyglutamine tracts is the autophagy-lysosome pathway [88]. We suggested that autophagy may be particularly relevant to aggregate-prone species [88]. Indeed, autophagy may be the natural default pathway for clearance of aggregated proteins, since recent data suggest that soluble, but not aggregated polyQ expansion-containing proteins, can be degraded by the proteasome [89]. This may arise because a protein must be unfolded before entering the central 20S proteolytic subunit of the proteasome complex. If redistribution of proteasomes into inclusions depletes the neuron of functional proteolytic activity, the results would be deleterious. This possibility is supported by the work of Kopito and colleagues, who showed that cells containing polyglutamine (or other) inclusions have impaired proteasome activities, compared to cells without aggregates [90]. A major role of the proteasome is to degrade short-lived proteins, like some transcription factors. The concentrations of some of these proteins needs to be carefully controlled, as they are critical regulators of cellular metabolism, hence the short half-lives. If the proteasome is impaired, the levels of such short-lived proteins rise abnormally and apoptosis often ensues.

PROPOSED MECHANISMS OF PROTEIN AGGREGATION

Three non-mutually exclusive mechanisms have been proposed to explain the aggregation of mutant huntingtin.

1) The Polar Zipper Model

One possibility is that the normal protein conformation is destabilized by the presence of the expanded polyglutamine tract, which, in turn, leads to abnormal protein-protein interactions and the formation of insoluble β-pleated sheets by linking β-strands together into barrels or sheets via hydrogen bonding, forming so-called polar zipper structures [91,92]. This model is supported by in vitro studies, which showed that purified recombinant proteins with glutamine expansions formed stable aggregates exhibiting polarisation with Congo Red, characteristic of proteins that have β-pleated sheets [93].

2) The Role of Transglutaminases

Aggregation of mutant huntingtin products has been postulated to be mediated by transglutaminases [94], enzymes normally involved in crosslinking of glutamine residues in different proteins. Huntingtin is a substrate of transglutaminase in vitro and the rate of the reaction increases with length of the polyglutamine tract [95]. An expanded polyglutamine stretch could result in increased cross-linking between mutant huntingtin and itself or other proteins and precipitation with slow intraneuronal accumulation of huntingtin aggregates. The activity of transglutaminases has been seen to be increased in HD patients compared to normal individuals [96], and it has been proposed that the level of transglutaminase activity affects the age of onset in individuals with equal numbers of CAG repeats [97]. However, tissue transglutaminase is not required and does not facilitate huntingtin aggregate formation [98], although this enzyme does appear to modify proteins that associate with truncated mutant huntingtin [99]. The data of Chun et al. [99] suggest that this modification is more likely to involve polyanimation than crosslinking. It is tempting to speculate that this phenomenon may play a role in HD pathology, but further work is required. A role for transglutaminase is aggregate formation is however supported by the observation of reduced aggregate formation and disease progression in an HD exon 1 transgenic mouse treated with the transglutaminase inhibitor cystamine [100]. These data do not allow one to draw conclusions about the pathogenic role of aggregates, as cystamine has been shown to block caspase activation [101].

3) Linear Lattice Model

A third model for aggregation may be simply that polyglutamine tracts are sticky and that longer polyQ stretches have a greater multiplicity of binding sites, allowing more intermolecular interactions [102].

THE TOXIC FRAGMENT HYPOTHESIS

It appears that full-length huntingtin may be cleaved to form an N-terminal fragment containing the glutamine repeats that is more toxic and which is also more prone to aggregation, compared to the full-length protein [103]. In HD knock-in mutant mice, there appears to be nuclear localization of full-length mutant huntingtin, followed by the formation of an N-terminal fragment and insoluble aggregate formation [104]. These changes show a repeat length-dependency and precede any clinical features of disease. Li et al. compared the patterns of inclusion formation in HD transgenic mice expressing only exon 1 with expanded repeats to knock-in mice expressing full-length mutant mouse huntingtin homologues [105]. The former develop inclusions in most brain regions, while the knock-in mice develop inclusions that are confined to the striatum [105]. While this study was not perfectly controlled, since the transgenes are expressed by different promoters (the mouse and human HD promoters differ [106]) and inclusion formation is not a direct measure of cell dysfunction/death, it supports the hypothesis that tissue-specificity of huntingtin proteolysis may be an important determinant of which cell populations are affected. The cleavage/toxic fragment model described above has recently been challenged by data suggesting that wild-type huntingtin is more susceptible to cleavage compared to mutant huntingtin [107]. While further work will be required to resolve this important controversy,
Hayden and colleagues have shown that huntingtin cleavage precedes neurodegeneration in a YAC transgenic mouse model expressing full-length mutant human huntingtin and that cleavage is apparent in early-stage human disease [108]. If cleavage is a rate-limiting step in HD pathogenesis, then cell-type differences in the complement of the relevant active proteases coupled with differences in gene expression may account for the differential cell-type vulnerability to the HD mutation, compared to the other polyQ diseases (some others which are also associated with cleavage of the mutant protein).

**CELL DEATH IN HD**

Apoptosis is programmed cell death – a conserved cellular mechanism initiated by diverse stimuli that leads to activation of aspartate-specific proteases (caspases), culminating in DNA fragmentation and cell death. A subset of neurons and glia in the neostriatum of post-mortem HD brains show DNA strand breaks (as assayed with terminal transferase-mediated deoxyuridine triphosphate–biotin nick-end labelling (TUNEL) [109-111]. Isolated medium spiny neurons were stained, most intensely in the putamen, followed by the globus pallidus and caudate. Labelling was also increased in more advanced cases of the disease. There appears to be a positive correlation between the number of CAG repeats in HD and the degree of nuclear fragmentation in the HD striatum [112]. However, TUNEL recognises some forms of necrosis and is thus not selective for apoptosis [113].

Apoptosis may not be the major mode of cell death in HD. Davies and colleagues found that the R6/2 HD transgenic mouse lines do develop late onset neurodegeneration within the anterior cingulated cortex, dorsal striatum, and in the Purkinje neurons of the cerebellum [114]. Dying neurons characteristically exhibited neuronal intranuclear inclusions, condensation of both the cytoplasm and nucleus, and ruffling of the plasma membrane while maintaining ultrastructural preservation of cellular organelles. These cells do not develop blebbing of the nucleus or cytoplasm, apoptotic bodies, or fragmentation of DNA. Neuronal death occurs over a period of weeks not hours. Degenerating cells of similar appearance were observed within these same regions in brains of patients who had died with HD. Thus, these workers suggested that the mechanism of neuronal cell death in both HD and a transgenic mouse model of HD is neither by apoptosis nor by necrosis [114]. However, a failure to observe apoptotic cells may be a function of their rapid clearance. Therefore, it is not clear which cell death pathway(s) are operating in HD.

Caspases are a family of cysteine proteases which are activated in apoptosis. A number of studies have suggested a role for these proteases in polyglutamine diseases. During apoptosis, caspase-3 cleaves structural and nuclear proteins, as well as other caspases [115]. Caspase-3 also specifically cleaves huntingtin and long polyglutamine sequences make huntingtin more susceptible to this cleavage [116]. In vivo studies suggest that N-terminal cleavage products of mutant huntingtin are more toxic and more prone to aggregate formation than the full-length protein. Toxic fragments from proteins with polyglutamine expansions may further activate caspases, which produce more toxic fragments in a positive feedback loop, ultimately resulting in cell death [117].

In a transgenic HD mouse model, expression of a dominant negative caspase-1 mutant that inhibits the activity of caspase-1, delays onset of symptoms and extends survival. Direct intraventricular administration of a caspase-1 inhibitor also delayed disease progression and mortality in the mouse model of HD [118].

Caspase 8 appears to be a necessary mediator of death in primary rat neurons transiently transfected with a construct expressing an expanded polyglutamine repeat [119]. This study showed that caspase 8 is recruited into inclusions and suggested that it is activated as part of the HD disease process.

Another pathway which may be relevant to HD pathogenesis is the activation of c-Jun amino-terminal kinases. This pathway is induced by a variety of oxidative stress stimuli and can induce apoptosis. Expression of mutant huntingtin with expanded polyglutamine repeats in rat hippocampal neuronal cells in vitro has been shown to stimulate JNKs activity and induce apoptotic cell death, whereas expression of normal huntingtin had no toxic effect. JNK activation preceded apoptosis and co-expression of a dominant negative mutant form of the stress signalling kinase (SEK1) almost completely blocked JNK activation and apoptosis [120].

**EARLY CHANGES IN GENE EXPRESSION**

A number of studies have used cDNA expression and Affymetrix arrays to interrogate early changes in gene expression in HD transgenic mice [121-125] and in stable inducible cell models [85]. The expression of many classes of genes that could participate in neurodegeneration are perturbed, including neurotransmitter receptors and proteins involved in different signalling pathways. One group of genes that are downregulated in mutant mice/cells are those that are controlled by cAMP response elements (CRE) [85,122]. A reduction of CRE-mediated transcription is also likely in human HD, since reduced levels of the CRE-responsive genes, somatostatin, corticotrophin releasing hormone, proenkephalin and substance P, are seen in HD vs. control brains, even in early stages of the disease [126-129]. Such genes may be relevant to the increased susceptibility to cell death and decreased neurite outgrowth seen in HD and related cell models, as these phenotypes can be partly attenuated by stimulating CRE-mediated transcription by overexpressing transcriptional co-activators like CRE-binding protein (CREB) or TAFII130, or by treating cells with cAMP or forskolin (which stimulates adenylyl cyclase) [85,130-132].

Decreased CRE-mediated transcription may also be important in relation to neurite outgrowth, which may impact on cell dysfunction that occurs in the early stages of polyQ diseases prior to cell death [85,133]. This may be relevant in vivo, since CREB-mediated signalling is crucial for long-term potentiation (LTP), the synaptic analogue for memory [134]. LTP is impaired in a number of HD mouse models and memory difficulties are a feature of HD [135, 136].
There are a number of potential non-mutually exclusive mechanisms that may act upstream of CREB in polyQ diseases. Decreased adenyl cyclase activity has been observed in HD transgenic mice [122]. It is possible that some of these changes may be mediated by the increased levels of protein phosphatase 2A suggested by our expression assays [85]. This enzyme can deacetylate phosphorylated CRE-binding protein (CREB) [137], one of the main transcription factors that bind to CRE elements. Another appealing model invokes the sequestration of coactivators like CREB-binding protein (CBP) and TAFII130 by mutant polyQ stretches into inclusions, as these co-activators are important positive regulators of CRE-binding protein (CREB)-mediated transcription. CBP has been observed in aggregates in spinobulbar muscular atrophy [130], HD and DRPLA [132, 138] and TAFII130 in SCA3 and DRPLA [131]. It may be difficult to unravel the relative importance of these different mechanisms.

Transcription in HD may also be more widely affected, as the mutant protein binds to the acetyltransferase domains of histone acetylases like CBP and p300 [139]. This is associated with impaired histone acetylation and this defect can be partially reversed in cell culture models by treating with histone deacetylase inhibitors. Histone deacetylase inhibitors slowed the progression of the disease in a Drosophila HD model [139] and have modest beneficial effects in the R6/2 mouse model [140], suggesting that such drugs may be beneficial in the human disease. Interestingly, histone acetylation appeared normal in the R6/2 mouse brains [140]. Thus, the histone deacetylase inhibitors may be protecting indirectly [141], as opposed to rescuing a pathway that is significantly compromised in HD.

Another class of genes that may be downregulated in HD are those controlled by Sp1, a transcription factor that binds to GC-rich elements in certain promoters and activates transcription of the corresponding genes. Li and colleagues [142] and Dunah et al. [143] showed that polyglutamine expansion increases the interaction of N-terminal huntingtin with Sp1. In HD transgenic mice (R6/2) that express N-terminal-mutant huntingtin, Sp1 binds to the soluble form of mutant huntingtin but not to aggregated huntingtin. Mutant huntingtin inhibited the binding of nuclear Sp1 to relevant target promoters and suppressed their transcriptional activities in cultured cells. Dunah et al. suggested that mutant huntingtin decreased the interactions between Sp1 and TAFII130 and that the combination of these two factors reduced the cellular toxicity of mutant huntingtin [143].

EXCITOTOXICITY AND IMPAIRED ENERGY PRODUCTION

Excitotoxicity refers to death of neurons as a result of exposure to excitatory amino acids, like glutamate. Excitotoxic pathways could be important modifiers of HD neuropathology, since animals injected intracranially with excitatory amino acids, such as kainate [144, 145] and quinolinic acid have similar striatal pathology to that seen in HD [146-148]. HD brains also show a loss of binding sites for excitatory amino acids [149], suggesting that cells expressing these receptors are made vulnerable by the mutant HD protein. The role of excitotoxicity in HD is further supported by the finding that genotypes at the GluR6 kainate receptor locus may modify the age-at-onset of symptoms independently of the effect of the CAG repeat number [18,19]. A contribution of excitotoxicity to HD pathology is supported by Zeron et al., who have shown that mutant full-length huntingtin enhances the excitotoxic cell death in HEK293 cells expressing NR1A2B N-methyl-D-aspartate (NMDA) glutamate receptors after treatment with 1mM glutamate [150]. This group have gone on to show that medium spiny neurons in a full-length mutant huntingtin YAC transgenic mouse are particularly prone to NMDA-mediated cell death, compared to wild-type animals. This effect could be abolished with an NR2B NMDA-receptor subtype specific antagonist [151].

Excitotoxicity in HD may be the result of a reduced threshold for glutamate toxicity that would occur in neurons with compromised energy metabolism, causing otherwise normal levels of this excitatory neurotransmitter become toxic [152,153]. A role for mitochondrial dysfunction in HD is further supported by recent work from Greenamyre and colleagues who reported that lymphoblast mitochondria from patients with HD have a lower membrane potential and depolarize at lower calcium loads than do mitochondria from controls [154]. Similar defects were observed in brain mitochondria from transgenic mice expressing full-length mutant huntingtin, and this defect preceded the onset of pathological or behavioral abnormalities by months. Impaired energy metabolism has been observed in the brains of HD patients using nuclear magnetic resonance spectroscopy [155], positron emission tomography [156] and using biochemical methods [157,158]. The relevance of impaired energy metabolism to HD pathology is also suggested by the effects of the toxin 3-nitropropionic acid (3-NP), which irreversibly inhibits succinate dehydrogenase, an enzyme involved in the tricarboxylic acid cycle and the electron transport chain during ATP synthesis. When 3-NP is administered chronically in low doses to animals it reproduces the slow progressive nature of human HD and the neuropathological and neurological outcomes closely mimic human HD [159,160]. Humans surviving 3-NP toxicity develop choreiform movements and dystonia [161,162].

Oxidative stress has been implicated in late onset neurodegeneration and there is in vivo support for oxidative damage to mitochondrial DNA in HD parietal cortex [163], evidence for free radical production in the brains of HD patients and transgenic mice [158, 164] and SOD-2 upregulation in an HD cell model [165]. We have recently observed that mutant huntingtin causes increased levels of ROS in neuronal and non-neuronal cells. Our data suggested that ROS contributed to cell death and was not simply a consequence of apoptosis because both N-acetylated-L-cysteine and glutathione in its reduced form suppressed polyQ-mediated cell death [166]. Interestingly, the polyQ expansion-dependent increase in ROS production and toxicity can be suppressed by HSP27. This hsp mediates its protection without reducing aggregate formation [166].

It is not clear if mitochondrial dysfunction and/or oxidative stress are primary changes or a consequence of the early neuropathological changes in HD. Guidetti et al. have
argued that mitochondrial changes may be secondary events, since measurements of mitochondrial electron transport Complexes I-IV did not reveal changes in the striatum and cerebral cortex in symptomatic HD transgenic mice without overt neuronal death [133]. The neostriatum and cerebral cortex in human presymptomatic and pathological Grade 1 HD cases also showed no change in the activity of mitochondrial Complexes I-IV. Nevertheless, mitochondrial dysfunction, even if it is a secondary event, may impact on the pathological processes.

**THERAPEUTIC STRATEGIES**

At present, there is no treatment that can arrest the course of HD. Recent trials in humans have used remacemide, a non-competitive NMDA receptor antagonist, and lamotrigine, which blocks voltage-gated sodium channels inhibiting glutamate release – these compounds were selected on the assumption that excitotoxicity plays an important role in HD [167,168]. Coenzyme Q10, an antioxidant and cofactor involved in mitochondrial electron transfer has also been tested on its own and in combination with remacemide [169-171]. Although these and previous trials using baclofen [172] idebenone [173] and vitamin E [174] showed no clear effects, it is possible small beneficial effects may have been missed. For instance, the recent “large” trial of remacemide and coenzyme Q10 was designed with power to detect a 40% slowing of functional decline in a 30 months period on early HD patients [168]. It is possible that such drugs may show clear beneficial effects if used for longer, or in larger studies, and may delay onset of disease if used in presymptomatic patients. Clearly, the logistical and cost implications of such large long-term trials in a comparatively rare disease will tend to restrict candidates to those which show major effects in symptomatic cases. Nevertheless, the failure to see effects with these compounds suggests they are not having very large effects. Hence, many are making major efforts to identify other approaches that may delay the onset of disease or slow its progress.

Another experimental approach that has been used to treat HD patients is striatal grafting. In rodents and non-human primates, striatal xenografts and allografts can survive, integrate into the host brain circuitry and improve motor and cognitive functions in animals subjected to metabolic/excitotoxic lesions that mimic some of the features of HD (reviewed by Beal and Hantraye [175]). Freeman and collaborators showed that grafted fetal striatal cells can survive and develop normally in the striatum of an HD patient [176]. Recently Bachoud-Levi et al. reported promising pilot data from a small trial of 5 HD patients who received striatal grafting. Three of the 5 patients appeared to show a beneficial response to the procedure, while no overt functional benefit was seen in the other two patients [177]. A number of centres are currently testing this procedure and it will be interesting to see data on a larger series of cases. It will be particularly important to investigate the long-term consequences of this procedure on dementia, as well as motor disturbances, since it is not clear to what extent striatal grafting could attenuate the loss of neurons in other regions of the brain in HD, like the cortex.

The availability of a number of different mouse models of HD has provided powerful tools for preclinical testing of therapeutic strategies, since mice have uniform mutations (similar CAG lengths) and genetic backgrounds. Furthermore, compounds can be tested in the animals prior to onset of disease and data can be accumulated fairly rapidly, given the short lifespan of many of the HD mouse models. Promising results have been reported with minocycline, a tetracycline derivative that inhibits transcription of caspases and the inducible form of nitric oxide synthase, among other pathways, and creatine, which can buffer energy levels [178,179].

Other approaches that may be successful are those which inhibit polyglutamine aggregation. Huntington aggregation and cell death have been reduced in cell models with human single chain Fv intracellular antibodies [180], a polyglutamine antibody IC12 [181], and peptides that interfere with polyglutamine aggregation [182].

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**REFERENCES**


