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Toward Understanding the Molecular Pathology of Huntington's Disease

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Huntington's Disease (HD) is caused by expansion of a CAG trinucleotide beyond 35 repeats within the coding region of a novel gene. Recently, new insights into the relationship between CAG expansion in the HD gene and pathological mechanisms have emerged. Survival analysis of a large cohort of affected and at-risk individuals with CAG sizes between 39 and 50 repeats have yielded probability curves of developing HD symptoms and dying of HD by a certain age. Animals transgenic for the first exon of huntingtin with large CAG repeats lengths have been reported to have a complex neurological phenotype that bears interesting similarities and differences to HD. The repertoire of huntingtin-interacting proteins continues to expand with the identification of HIP1, a protein whose yeast homologues have known functions in regulating events associated with the cytoskeleton. The ability of huntingtin to interact with two of its four known protein partners appears to be influenced by CAG length. Caspase 3 (apopain), a key cysteine protease known to play a seminal role in neural apoptosis, has also been demonstrated to specifically cleave huntingtin in a CAG length-dependent manner. Many of these features are combined in a model suggesting mechanisms by which the pathogenesis of HD may be initiated. The development of appropriate in vitro and animal models for HD will allow the validity of these models to be tested.

Introduction

Huntington's Disease (HD), an inexorably fatal inherited neurodegenerative disorder for which there is currently no treatment, normally strikes in adulthood with the onset of uncontrolled movements, altered behavior, and cognitive decline (74). The past few years have been an exciting period of research on HD beginning with the cloning of the causative gene in 1993 (81). Since then significant progress has been made on many fronts. These include the classification of HD as a triplet repeat disorder caused by expansion of a CAG trinucleotide encoding polyglutamine, a deeper understanding of the correlations between CAG size with the age of onset and age of death in HD patients, the identification of several proteins that interact with huntingtin, and tantalizing associations between huntingtin with expanded CAG repeats and factors associated with apoptotic cell death. The advances in these areas are the subject of this review.

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Genetic considerations in HD patients

HD belongs to a group of eight disorders with several remarkably similar features (Table 1). For example, the genetic defect in each of these diseases is expansion of a CAG trinucleotide in the coding region of their respective genes that must be translated into polyglutamine to result in the disease phenotype (61, 83). All of these diseases result in extraordinarily selective patterns of neurodegeneration even though the causative gene products are widely expressed (147). Each of these trinucleotide repeat disorders is inherited as an autosomal dominant disorder except for spinal and bulbar muscular atrophy (SBMA) which is X-linked (100). None of the gene products of these diseases have known functions or substantial homology with each other, except for their CAG tracts, or any other known protein in GenBank, except for SBMA which encodes the androgen receptor (100) and for spinocerebellar ataxia type 6 (SCA6) which encodes a P-type α1A voltage-dependent calcium channel subunit (187).

Taken together,the similar underlying mutation within novel genes raises the possibility that the fundamental mechanisms underlying pathology in the polyglutamine triplet repeat diseases may have elements in common. It remains a major research challenge to identify the similarities in pathways leading to cell death in this group of diseases while also providing explanations for the distinct patterns of selective neuronal vulnerability characteristic of each disorder.

Disease	DRPLA (Dentatorubro-pallydoluysian atrophy)	HD (Huntington's Disease)	MJD/SCA3 (Machado-Joseph disease/ spinocerebellar ataxia type 3)	SBMA (Spinal and bulbar muscular atrophy)	SCA1 (Spinocerebellar ataxia type 1)	SCA2 (Spinocerebellar ataxia type 2)	SCA6 (Spinocerebellar ataxia type 6)	SCA7 (Spinocerebellar ataxia type 7)
CAG repeat gene product	Atrophin CTG-B37	Huntingtin IT-15	MJD1	Androgen receptor	Ataxin-1	Ataxin-2	α1a-voltage-dependant calcium channel	Not cloned
Normal CAG repeat size	3-35	6-35	12-40	11-33	19-36	15-29	4-16	
Disease CAG repeat size	49-85	36-121	62-84	38-66	41-81	35-59	21-27	
Region of neurodegeneration	Dentatorubral and pallidoluysian systems	Caudate/putamen	Dentate nucleus; substantia nigra, pontine nucleus; subthalamic nucleus; spinocerebellar tracts	Anterior horn; primary sensory neurons; V, VI, VII, XIIcranial motor nuclei	Purkinje neurons; inferior olive	Purkinje neurons; inferior olive substantia nigra	Purkinje neurons inferior olive	
Normal Protein Size (kDa)	190	348	42	76	87	140	252	130

Table 1. Neurodegenerative diseases involving expansions of CAG repeats within novel genes.

Clinical and neuropathological features of HD

Patients with HD are afflicted with uncontrolled movements, altered behavior, and cognitive decline (73). The motoric features of HD include prominent, involuntary choreiform movements, dystonia and speech disturbances (71, 73). Cognitive impairment in HD patients includes problems in recall, organization, and acquisition of new information (71, 73). Depressive disorders are frequent in HD patients and psychotic symptoms resembling schizophrenia may also develop in a minority of patients (71, 73).

Neuropathology in HD is characterized chiefly by a selective and progressive neurodegeneration of the basal ganglia by mechanisms that are not as yet completely understood. The characteristic neuropathological lesion in HD is the selective loss of GABAergic medium-spiny neurons with associated gliosis in the neostriatum (65, 96, 144). In contrast, striatal interneurons containing somatostatin and neuropeptide Y are largely spared (11). Interestingly, these interneurons also stain positive for NADPH diaphorase (36, 56), raising the possibility that the ability to produce neuronal nitric oxide synthase (nNOS) may be neuroprotective in HD (78).

Pathological severity in autopsy cases of HD has been classified into 5 grades, ranging from grade 0 (i.e. no identifiable pathology in cases with premortem symptomology) to grade 4 (i.e. severe atrophy, neuron loss and astrocytosis in the neostriatum) (175). From grades 1-4 the generalized loss of medium neurons proceeds in a dorsomedial-to-ventral gradient beginning in the body and tail of the caudate and later extending to the external segment of the globus pallidus (96, 175). However, isolated islands of neuronal loss and astrocytosis within the striosome compartment of the neostriatum have been reported in autopsy material even from grade 0 cases (75). This observation leads to the suggestion that the early degeneration of striosomal neurons may produce hyperactivity of the nigrostrial dopaminergic pathway, resulting in chorea and other early clinical signs of HD (75).

There is selectively decreased immunoreactivity for enkephalin in the external pallidum at least through grade 2, implicating a loss of axonal projections from a specific subset of GABAergic striatopallidal neurons (1, 102, 145). Disinhibition of neurons in the external pallidum, resulting from a loss of axonal projections from a loss of inhibitory enkephalin-immunoreactive striatopallidal axons, could produce both increased activity in the pallidoreceptive thalamocortical neurons and chorea.

Morphometric analyses of autopsy material from cases with advanced grades of HD (101) have reported significant neuronal loss in the striatum. Neurodegeneration may also be found in both segments of the pallidum and the medial and lateral compartments of the subthalamic nucleus. In the cerebral cortex there is a loss of neurons and resulting astrocytosis, mainly in layers III-V (169). Importantly, neuronal degeneration appears to result from apoptosis (141, 171).

In vivo lesion studies demonstrate that the pattern of cell death in HD can be reproduced by glutamate receptor agonists that act on the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors (6, 9). Furthermore, because cultured striatal neurons are susceptible to

NMDA-receptor mediated toxicity (59), it has been suggested that striatal neurons in HD may die as a result of glutamate-induced excitotoxicity.

Correlations between CAG length and the phenotype of HD

Age of Onset. Although the clinical features of these diseases normally appear in adulthood and progress inexorably through stages of increasingly severe disability, it has been well established that the age of onset is variable with younger patients typically exhibiting a more severe clinical course (85, 89, 90, 99, 109, 114, 127, 142, 168). Several studies describing the relationship between CAG size and the age of onset of HD have focused on affected persons and have had various interpretations of the range of CAG repeat sizes on normal and HD chromosomes (3, 5, 13, 29, 34, 39, 44, 60, 81, 103,110, 134, 148, 159, 162, 165, 172, 188). However, these prior studies have not included asymptomatic, at-risk individuals with CAG length in the affected range. This omission makes it impossible to take into account the number of asymptomatic persons with a particular CAG at a specific age, and thus prevents a complete understanding of the relationship between CAG size and age of onset of HD.

As the range of age of onsets for a particular CAG repeat size in the HD gene is very broad, most authors including ourselves have recommended against using the CAG repeat size to predict the age of onset for an individual patient (3, 5, 34, 39, 44, 110, 134, 135, 156, 165, 172). In 1994, the Huntington Study Group, a consortium of investigators and practitioners across North America, defined the ranges of CAG repeat length for normal persons as less than 30, while a CAG of 38 or greater indicated a high probability of developing HD (80). A repeat size of 30 to 37 was considered indeterminate as to whether the patient would develop HD at some time in the future. These guidelines were put in place pending more definitive information concerning the relationship between CAG length and clinical phenotype of the disease.

A recent analysis of a large cohort of affected and asymptomatic at-risk persons with CAG expansion allows the development of estimates of the probability of developing HD by a particular age with a given CAG size (19). This cohort numbered 1,049 persons, including 321 at-risk and 728 affected individuals with a CAG size between 29 and 121 repeats. Of these, 866 individuals (90%) from 445 families had CAG repeat lengths between 39 to 50 repeats. For each year of age and CAG size in this range, all individuals, including both those asymptomatic at-risk and those affected, were taken into account to calculate the cumulative probability of having onset of HD by that age by using Kaplan-Meier survival analysis. Including asymptomatic at-risk as well as affected individuals has allowed development of survival curves that provide probabilities of becoming affected with HD by a certain age (Figure 1, Table 2). These results show that for individuals 45 years of age, for example, the probability of developing HD is 13% for those with 40 CAG repeats, 32% for someone with 42 CAG repeats, 73% for 44 repeats and 100% for a person with 46 CAG repeats (Figure 1). It is noteworthy, for example, for a CAG of 45, 100% of persons in this study were affected by 48 years. Similar estimate can be made for different CAG lengths (Table 2).

CAG Repeat Size	Latest Reported Age of Onset	Number of Individuals
36	65	1
37	60	4
38	84	2
39	71	8
40	84	64
41	75	74
42	65	98
43	65	92
44	58	95
45	48	63
46	45	58
47	45	39
48	45	31
49	45	26
50	34	13

Table 2. Latest age by which persons had documented age of onset for a particular CAG size in this series.

While these probability curves cannot be used to predict the particular age of onset for an individual, this analysis may have clinical utility by providing estimates of symptom-free survival to an individual seeking additional information in a predictive testing program. Data generated from this study may also have significant implications for the design of clinical trials for new therapeutics. Appropriate design of clinical trials will need to take into account the expected age of onset of HD for a particular person to determine the potential efficacy of therapy. For example, all persons with a CAG length of 46 repeats would expect to manifest by 45 years of age with a median age of onset of 36. Extension of age of onset beyond this age could indicate a significant therapeutic effect of a particular drug.

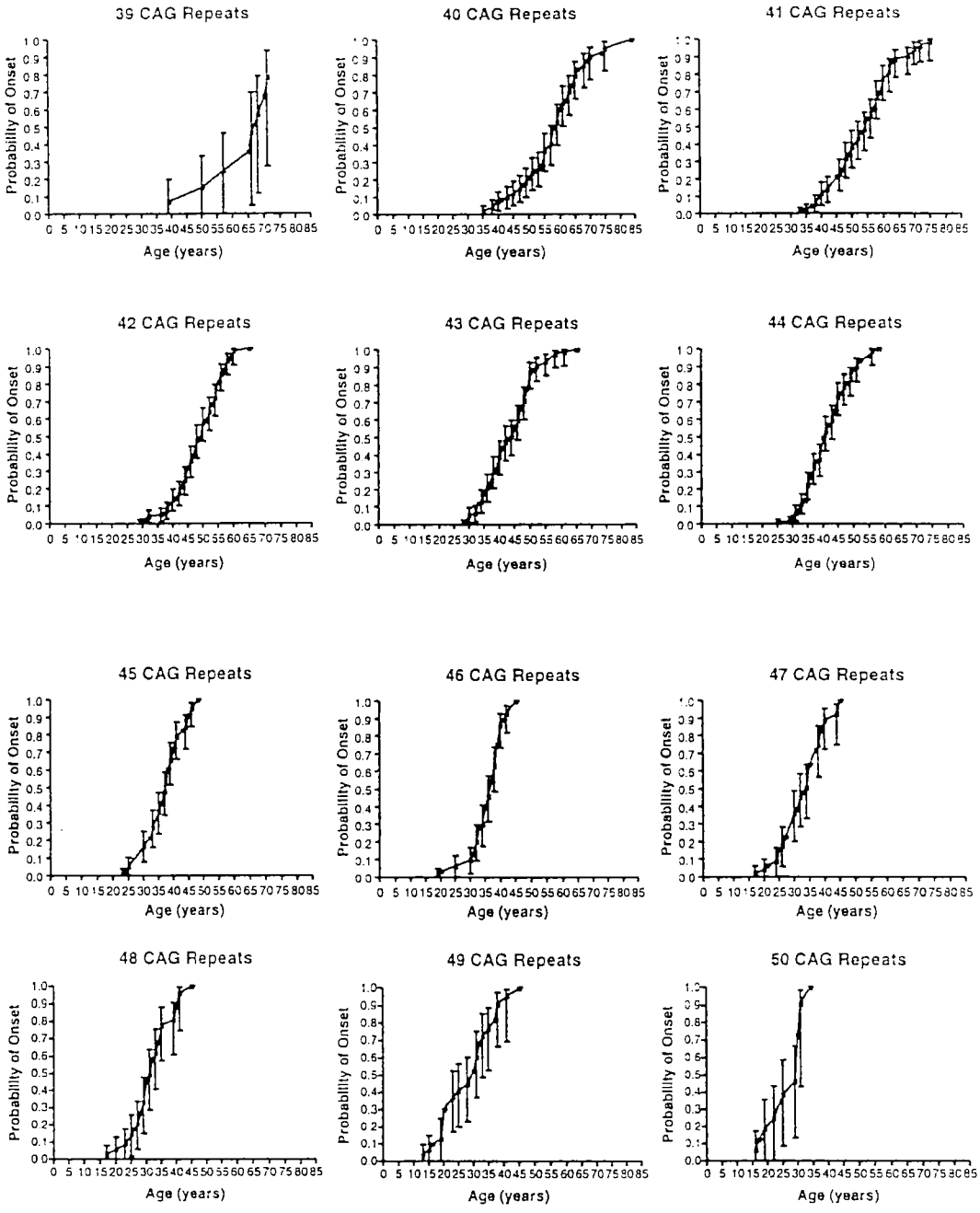

Figure 1. Graphs represent the cumulative probability of being affected at a particular age for a CAG repeat size between 39 and 50. Error bars represent 95% confidence intervals.

Alternatively, shifting of the median age of onset beyond 36 might also indicate a therapeutic effect. These data indicate the importance of having sufficient persons with a particular CAG size in a drug trial to allow for more rapid ascertainment of a beneficial effect.

Reduced penetrance in HD. HD has previously been considered to be 100% penetrant (71, 73, 82), but it is now appreciated that penetrance is modified by CAG size (19, 129, 149). Using onset greater than or equal to 75 for males and greater than or equal to 81 for females as beyond the normal lifespan, analysis of survival curves indicates that there is complete penetrance with a CAG repeat size of 42 or above (19). Reduced penetrance may occur within the range of 36 to 41 CAG repeats as clinical presentation of HD does not always occur within the expected mean life span of an individual. There is a trend to increasing penetrance with increasing repeat length in the 36 to 41 repeat range, up to 90% for 39 CAG repeats and 99% for 41 CAG repeats (19).

Age of Death. In addition to predicting age of onset, survival analysis has also been used to determine the probability of death by a particular age for an individual with a specific CAG size (Brinkman and Hayden, unpublished data). Individuals with CAG > 36 repeats were selected from the cohort used in the previous study (19) and the data subjected to Kaplan-Meier survival analysis. We included 542 individuals with CAG lengths of 41-45, of which 75 had died. The majority (60%) of persons in our database with a CAG size of ≥ 36 have a CAG repeat in this range. Other CAG lengths were excluded from the survival analysis, since the small numbers of individuals for these particular CAG sized precluded rigorous statistical analysis.

The resulting Kaplan-Meier curves are all significantly different from each other ($P < 0.05$) except for the 43 and 44 CAG curves ($P < 0.5$). For example, while only 4% of persons with 41 CAG repeats (n = 74) had died by 60 years of age, this increased to 30% for 43 CAG repeats (n = 93) and 82% for 45 CAG repeats (n = 66). This data, along with the age of onset analysis, shows the dramatic and overriding effect of CAG repeat length on the age-specific phenotype of individuals with HD.

Gain, loss, or altered function due to polyglutamine expansion in triplet repeat diseases. Several models have been proposed to explain how genes with expanded polyglutamine tracts cause disease. Gene knockout studies for HD are most compatible with a gain-of-function model. Three groups have generated targeted disruptions in Hdh, the murine homologue of huntingtin. While each group in different regions disrupted expression of Hdh, all observed lethality at approximately E8.0 days of gestation for mice homozygous for the targeted mutation (45, 131, 186). Hdh-null mice have features of increased apoptosis and appear unable to elaborate the extraembryonic mesoderm(186). The early embryolethality observed in these studies argues against models proposing that HD is caused solely by loss of function associated with CAG expansion or by dominant-negative mechanisms whereby the mutant allele interacts with and inactivates the normal gene product. The strongest evidence against loss of function being causative of HD is that individuals homozygous for expanded alleles are viable and do not appear to have a more severe phenotype than persons heterozygous for CAG expansion (125, 180).

When the polyglutamine expansion diseases are viewed as a group, however, mechanisms that suggest that disease results from altered or loss of function cannot be completely excluded. For example, the gene product in SCA6 is a mutant P-type α1A calcium channel, a protein that is known to be important for Purkinje cell survival (106, 121). In SCA6, CAG expansion results in progressive cerebellar and brain stem neurodegeneration (187), while patients with the allelic disorders hereditary paroxysmal cerebellar ataxia (HPCA) and episodic ataxia (EA) have mild and intermittent cerebellar dysfunction that is due to protein truncation of this calcium channel (97, 173). Similar mutations in the murine homologue of the α1A calcium channel have been described that lead to ataxia and cerebellar degeneration only in animals with mutations in both alleles (57). The murine data, taken together with the observation that the α1A calcium channel is known to contribute to Purkinje cell function, suggests that the dominant neurodegenerative disorder associated with CAG expansion in SCA6 results not from the gain of a completely novel function but rather from the interference with normal channel activity. For example, CAG expansion may affect neurotransmitter release, or cause constitutive activation of the channel, resulting in abnormal levels of intracellular Ca^{2+}, and ultimately neurodegeneration (187).

CAG expansion in the androgen receptor gene may also result in a partial loss of receptor function in SBMA. Patients with SBMA exhibit neurodegeneration in the brain stem and spinal cord and also show a mild

decrease in androgen receptor activity (100). In contrast, the clinical outcome for individuals with deletions of one androgen receptor allele is gynecomastia and reduced fertility due to androgen insensitivity but with no accompanying neural cell loss (76). These observations argue that while neurodegeneration in SBMA appears to be due mainly to a gain of function, the polyglutamine expansion can also affect the normal function of the androgen receptor.

These observations suggest that polyglutamine expansions may interfere with the normal function of the gene product. These alterations may be a partial loss of function as seen in SBMA, or they may be more compatible with a dominant-negative effect as suggested for SCA6. In HD, the available data are most supportive of a gain of function model. However, we can not entirely exclude models suggesting that the mutant huntingtin gene product is particularly impaired in carrying out its normal function.

Mechanisms of toxicity in polyglutamine expansion diseases. Two major hypotheses have been developed to explain how proteins with expanded CAG tracts lead to neurodegeneration. One suggests that the polyglutamine expansion results in altered protein-protein interactions that manifest as gain of a novel property, loss of normal function, or deregulated activity. The other hypothesis suggests that polyglutamine expansion results in the production of toxic protein fragments. Because altered protein-protein interactions may indirectly lead to generation of toxic protein fragments, these hypotheses are not mutually exclusive.

Proteins interacting with huntingtin. Most of what is known about proteins that interact with products of trinucleotide expansion genes has come from studies with huntingtin. Two major and overlapping questions have provided considerable impetus for identification of proteins that interact with huntingtin. The first question relates to the attempt to understand the specificity of neuronal loss in HD, given that huntingtin is expressed in many tissues. The second question deals with molecular mechanisms of degeneration in cells with expanded CAG alleles. Four proteins that interact directly with huntingtin have now been described (22, 86, 87, 94, 104, 179). All of these proteins have been identified using either amino-terminal portions of huntingtin containing the polyglutamine tract in yeast two-hybrid screens, or by searching for proteins that interact directly with the polyglutamine tract itself using biochemical methods. Because the polyglutamine tract is in the amino-terminal portion of huntingtin, searching for proteins that interact in this region is reasonable. However, it should be remembered that huntingtin is a large protein of 348 kDa and is likely to have an expansive repertoire of interacting proteins. Characterizing the molecular interactions between huntingtin and its various partners will likely continue to be a fruitful area of research for several years.

HIP1

The most recently identified huntingtin-interacting protein is HIP1 (huntingtin-interacting protein 1). Two groups independently identified HIP1 in a yeast two-hybrid screen using an amino-terminal portion of huntingtin as bait (87, 179). Two features make HIP1 a particularly interesting protein. Not only does the interaction between HIP1 and huntingtin appear to be modulated by CAG length (87), but HIP1 appears to be the human homologue of a yeast protein with known roles relating to membrane and cytoskeleton functions. Thus, HIP1 represents a molecular link between HD and events at the membrane cytoskeleton and opens new areas for further study of how huntingtin alleles with expanded CAG tracts lead to neurodegeneration.

HIP1 maps to a single genomic locus at 7q11.2 (87). Northern blot analyses show that the approximately 9 kb HIP1 mRNA is expressed in all tissues studied but is enriched in the brain (87). In situ hybridization experiments confirm expression of HIP1 mRNA in striatum (87). There is some discrepancy concerning the degree of tissue specificity of HIP1 protein expression. Whereas Kalchman et al. (87) show that HIP1 protein is undetectable or present at very low levels in peripheral tissues, Wanker et al. (179) find HIP1 protein in liver, kidney, and heart at approximately half the levels that are detected in brain. This discrepancy may be due to differences in the epitope recognized by the different antibodies used in these studies. Alternatively, there could be family of HIP1 proteins with different degrees of tissue specificity.

Within the brain, Kalchman et al. (87) show that the highest levels of HIP1 immunoreactivity are observed in the cortex with lower levels seen in cerebellum, caudate and putamen. Because HIP1 is detectable in cerebellum, the interaction between HIP1 and huntingtin cannot fully account for the specificity of neurodegeneration in HD patients as the cerebellum is infrequently involved in the pathology of HD. Additional molecular partners, or cell-specific post-translational modifications, may also play a role. For example, the striatum could specifically contain other factors that interact with

the huntingtin-HIP1 complex and contribute to the specificity of neurodegeneration in HD. Alternatively, there may be proteins specific to striatal or cerebellar neurons that regulate the interaction between huntingtin and HIP1. Additionally, the molecular consequences of altered interaction between HIP1 and mutant huntingtin may be particularly detrimental to striatal neurons. Clearly, further studies are necessary to elucidate the function of the huntingtin-HIP1 interaction and its role in the pathology of HD.

Subcellular fractionation experiments show that HIP1 immunoreactivity is found in fractions containing cell debris and nuclei, mitochondria and synaptosomes, and microsomes and plasma membrane (87, 179). Although HIP1 is membrane associated, it does not appear to be integral since it can be solubilized by high salt washes (87). Biochemical fractionation experiments show that HIP1 is Triton X-100 insoluble, suggesting that it is a cytoskeletal-associated membrane protein. Additionally, HIP1 and huntingtin colocalize in all membrane fractions suggesting that they have the potential to interact at the membrane (87, 179). Immunohistochemistry and immunofluorescence experiments show that HIP1 is excluded from the nucleus with a non-uniform punctate staining in the cytoplasmic, further supporting the association of HIP1 with intracellular membranes (87).

The interaction between HIP1 and huntingtin in vivo was confirmed by demonstrating that HIP1 could be specifically coimmunoprecipitated from a human brain lysate using a monoclonal antibody specific for huntingtin (87). Recombinant histidine-tagged HIP1 has also been shown to be specifically retained by a huntingtin-GST fusion protein in in vitro binding experiments (179).

Results from a HIP1 deletion analysis using the yeast two-hybrid system show that the amino-terminal portion of HIP1 is sufficient for interaction with huntingtin (179). Interestingly, a strong interaction between HIP1 and huntingtin appears to require amino acids downstream of the huntingtin polyglutamine tract, as Wanker et al. (179) observe only weak interactions between HIP1 and a small huntingtin protein truncated immediately after the polyglutamine tract. Alternatively, conformational changes in the protein due to deletion of specific residues may account for these findings.

The region downstream of the polyglutamine tract in huntingtin contains HEAT repeats, motifs that are proposed to adopt a structure containing two α helices (2). Interestingly, HEAT repeats are found in several cytoplasmic regulatory proteins with known roles in transport processes, including VP15 that is involved in vesicle mediated protein transport, importins that are involved in nuclear protein import/export pathways, and PSE1 that is involved in protein secretion (2). These sequences may therefore be important in transport of huntingtin between different compartments in the cytosol.

The sequence of HIP1 shows distinct homology to the membrane cytoskeletal associated protein Sla2p in Saccharomyces cerevisciae and to a protein of unknown function in Caenorhabditis elegans designated ZK370.3 (77, 87). Genetic studies in yeast have shown that Sla2p has three known functions. Null mutations of SLA2 cause temperature-sensitive growth defects that presumably arise from a general disorganization of the membrane cytoskeleton. This mutation is lethal when combined with actin mutants deficient in binding fimbrin and ABP1 (actin-binding proteins) or when mated to ABP1 deletion mutants (77). Other studies show that the SLA2 gene product is required for normal endocytosis (123) and that it plays a role in regulating the abundance of yeast plasma membrane H+-ATPase, which is structurally and functionally similar to mammalian cation translocating P-type ATPases including Na+K+, H+K+ and Ca2+ATPases (126).

These studies suggest that the yeast homologue of HIP1 is involved in regulating events at the membrane through association with the cytoskeleton. What could these events be in the human brain? It is tempting to speculate that events at membrane-bound cytoplasmic organelles are prime targets and that HIP1 may function in the transport, assembly, or regulation of cytoplasmic organelles or in vesicle trafficking. Such functions may then be adversely impacted via aberrant interactions with huntingtin carrying expanded CAG repeats.

HAP1

Huntingtin- associated protein 1 (HAP1) is another protein identified by yeast two-hybrid screening whose interaction with huntingtin is modulated by CAG length (104). In contrast to HIP1, the interaction between HAP1 and huntingtin is increased with increasing CAG length. Semiquantitative liquid β-galactosidase assays show that HAP1 has increased affinity for huntingtin alleles with 44 glutamines, as opposed to an allele with 23 glutamines (104). HAP1 does not interact with an atrophin-1 construct containing 21 CAG repeats, suggesting that huntingtin sequences surrounding the polyglutamine tract are necessary for the interaction with HAP1 (104). Because both HIP1 and HAP1 bind to sequences within the amino-terminal portion of hunt-

ingtin, yet their binding affinities are affected by CAG length in reverse directions, it will be interesting to determine if HIP1 and HAP1 compete for overlapping binding sites.

In yeast, the first 230 amino acids of huntingtin is sufficient for an interaction with HAP1 (104). In vitro binding experiments with a glutathione S-transferase (GST)-HAP1 fusion protein have demonstrated that both endogenous huntingtin and a transiently transfected huntingtin construct of 930 amino acids are specifically bound by GST-HAP1 (104). Binding experiments using extracts from lymphoblastoid cell lines derived from HD patients confirm the correlation between repeat length and HAP1 interaction, as strongest binding is observed using extracts containing huntingtin alleles with 82 repeats. Huntingtin alleles with 44, or 22 or 19 repeats have progressively weaker interactions with HAP1 (104). Coimmunoprecipitation experiments show that HAP1 can be precipitated with an antibody specific for HD from either whole rat brain or from HEK293 cells transiently transfected with HAP1 (104). However, similar experiments with human brain tissue have so far been unsuccessful.

Northern blot analysis shows that expression of the 4.0 kb HAP1 mRNA is limited to the brain, with maximal expression in the rat striatum and olfactory bulb and weak expression in spinal cord, cerebellum, and cerebral cortex (104). A partial human homologue of HAP1 is expressed maximally in caudate, subthalamic nucleus, cortex, and fetal brain. Interestingly, a variety of human HAP1 cDNA homologues were detected, suggesting the existence of multiple forms of human HAP1 or the presence of a family of HAP1-like transcripts. Western blot analysis of HAP1 from monkey and human brain regions confirms that HAP1 is selectively expressed with high levels observed in hippocampus, caudate, and cortex and lower levels in cerebellum (105). Interestingly, although striatal neurons were found to contain low amounts of HAP1 mRNA, they react strongly with HAP1 antibodies in immunohistochemistry experiments (105). Because the expression pattern of HAP1 does not correlate precisely with the regions selectively involved in HD, additional factors must once again be invoked to account for the selective neurodegeneration in HD.

Subcellular fractionation experiments showed that HAP1 is predominately a membrane-associated protein that shows a subcellular distribution profile similar to synaptophysin, suggesting that HAP1 associates with the cytoskeleton or synaptic vesicles (105). Immunohistochemistry experiments show that HAP1 is exclusively cytoplasmic and associates with cytoplasmic granules with no apparent staining of the plasma membrane (105).

Although the primary sequence of HAP1 gives no clues as to its function, a combination of in situ and subcellular fractionation experiments have shown that HAP1 is expressed in several similar brain regions as neuronal nitric oxide synthase (nNOS) or NADPH diaphorase, an enzyme involved in the synthesis of nitric oxide. In particular, HAP1 and nNOS coexpression occurs in several regions including the accessory olfactory bulb, the supraoptic nucleus, pedunculopontine nucleus, large striatal neurons, Islands of Calleja in the basal forebrain, dentate gyrus, the CA1 hippocampal region, and the superior and inferior colliculi (105). The notable exception to colocalized staining was observed in the cerebellum, with the granule cells staining intensely for nNOS and NADPH diaporase while HAP1 staining was much lighter (105).

The correlation between HAP1 and nNOS is intriguing, as there is evidence that neuronally derived NO may play a role in excitotoxic mechanisms. For example, mice with targeted disruptions in the gene for nNOS, or that are treated with NO inhibitors, are substantially more resistant to excitotoxic damage caused by intrastriatal injections of malonate or the systemic delivery of 3-nitroproprionic acid (3-NPA) (37, 151). Because huntingtin alleles with expanded CAG tracts show increased binding to HAP1, this enhanced interaction may increase the synthesis of NO or result in aberrantly localized NO expression and render certain populations more susceptible to excitotoxic damage (105).

HIP2

A third huntingtin interacting protein identified using a yeast two-hybrid screen is HIP2 (86). Sequence analysis showed that HIP2 encodes the human ubiquitin conjugating enzyme, hE2-25K (27). The identity of HIP2 as hE2-25K was confirmed by demonstrating that GST-HIP2 reacts strongly with affinity purified antibodies raised against bovine E2-25K, while GST alone does not react (86). E2 ubiquitinating enzymes have known roles in the turnover of abnormal proteins (27, 40). It is noteworthy that altered patterns of cellular ubiquitination have been observed in other neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (150, 155, 166).

Similar to HIP1 and HAP1, binding of HIP2 to huntingtin requires sequences within the first 540 amino acids of huntingtin (86). Deletion analysis shows that the 5' boundary of the HIP2 binding site must lie prior

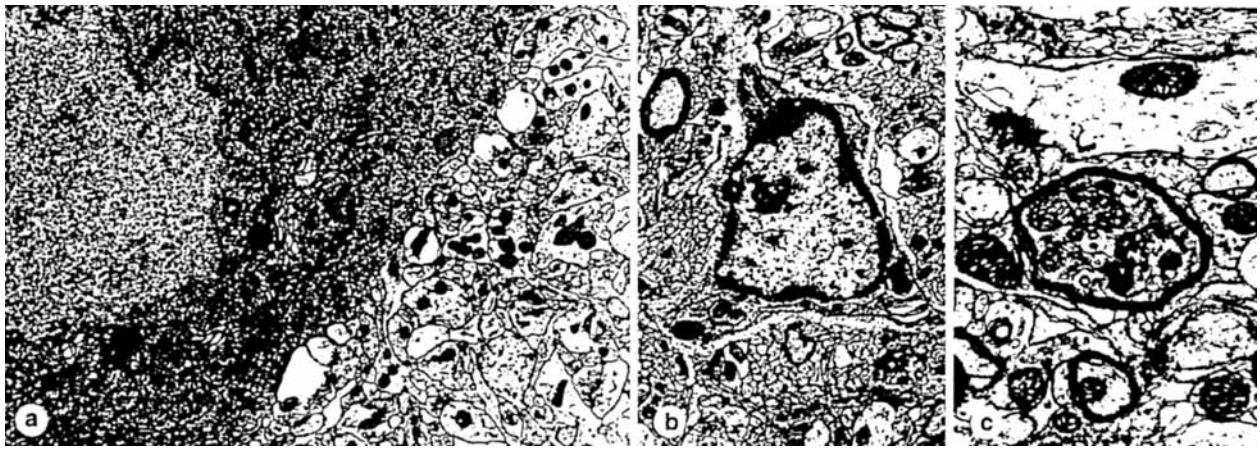

Figure 2. Electron micrographs of apoptotic neurons in the basal ganglia of mice at four months of age, heterozygous for the Hdhex5 mutation. **a.** Degenerating neuron in the caudate-putamen exhibiting the increased clumping of nuclear chromatin and overall condensation of the cytoplasm with protrusions of the cell surface, characteristic of early apoptosis. Individual organelles exhibit essentially normal morphology with increased packing density and electron density. Note that the mitochondrial profiles are not swollen in this neuron. Occasional axosomatic synapses (arrow) were observed on the cell surface (x15,545). **b.** Apoptotic neuron in the globus pallidus illustrating coarse chromatin clumping and peripheral margination of chromatin, charactertistic of a more advanced stage of apoptosis. The remnants of an axosomatic synapse were observed on the cell surface (arrow). Note the condensation of organelles and the relatively homogeneous dispersion of ribosomes in the cytoplasm of this neuron (x13,618). **c.** Myelinated axon in the subthalamic nucleus containing condensed mitochondria, clear vacuoles and electron opaque granular masses. These degenerating axons were relatively rare (i.e. 1-2 profiles per 10,000 μm2 of section area), but could be detected in the neuropil of the globus pallidus and subthalamic nucleus (x35,177).

to amino acid 125 and the 3' boundary must lie beyond amino acid 242 of huntingtin. However, in contrast to HIP1 and HAP1, the interaction between HIP2 and huntingtin does not appear to be obviously influenced by CAG length when tested with alleles carrying 16 or 44 repeats (86). It will be informative to test whether the binding of HIP2 is altered with polyglutamine lengths of 80 or 128 repeats.

The interaction between HIP2 and huntingtin was confirmed in vivo by demonstrating that endogenous huntingtin could be affinity purified from cell lysates using GST-HIP2 (86). Also, huntingtin is specifically detected in ubiquitin conjugates prepared from lysates of transformed lymphoblasts derived from an individual heterozygous for HD, showing that huntingtin is ubiquitinated in vivo (86).

E2-25K mRNA transcripts of 1.2 and 2.4 kb are expressed in all assessed human tissues and a 25 kD protein detected by E2-25K specific antibodies is ubiquitously expressed (86). However, the 25 kD band is enriched in brain. Interestingly, two higher molecular weight immunoreactive species of 28 and 45 kD are also detected in brain that may represent modified E2-25K proteins, additional members of the E2-25K family, or cross-reactive proteins. The expression profile of the 28kD band shows a striking parallel to the regional neuropathology in HD, being specifically enriched in striatum and cortex (86).

The correlation between selective expression of the presumed 28 kD form of E2-25K and the specific neuronal loss in HD is especially intriguing. If this 28 kD band turns out to be a form of E2 that is involved in the regulated catabolism of huntingtin, it is tempting to speculate that ubiquitination may play a role in the neuropathology of HD. For example, the number of ubiquitin reactive neurites in HD brain is increased compared to controls (24). This observation leads to the suggestion that ubiquitinated huntingtin may be degraded giving rise to toxic protein fragments particularly when polyglutamine expansion is evident (see below).

GAPDH

Glyceraldehyde-phosphate dehydrogenase (GAPDH) is a multifunctional enzyme with roles in glycolysis (5872) and in DNA repair and replication (119146). GAPDH has been reported to interact with microtubles (79160), actin (117), RNA (128157), and amyloid precursor protein (152). Using affinity chromatography methods, GAPDH has been shown to interact directly with the polyglutamine tracts of huntingtin and DRPLA (22), and genetic methodology employing the yeast two-hybrid method shows interactions

between GAPDH and the ataxin-1 and androgen receptor gene products (94).

The portion of GAPDH that was found to interact with ataxin-1 and androgen receptor contained amino acids 1-149 of the nicotinamide adenine dinucleotide (NAD) binding domain and only the first 21 amino acid residues of the catalytic domain, suggesting that the NAD binding domain is primarily involved in the interaction (94). It is not known whether the interaction of ataxin-1 or androgen receptor with GAPDH disrupts the function of GAPDH, possibly by preventing the formation of active tetramers.

There is some discrepancy as to whether the interaction of GAPDH with the products of the trinucleotide repeat genes is modulated by CAG length. In yeast two-hybrid studies, CAG length does not affect binding (94). However, in biochemical experiments using pure polyglutamine stretches immobilized on a solid support, GAPDH preferentially bound long lengths of polyglutamines (22). Further experiments will be necessary to reach a consensus on whether polyglutamine length as well as flanking sequences influences the strength of interactions with GAPDH. However, it is particularly interesting that GAPDH appears to interact preferentially with smaller fragments of huntingtin as opposed to the full-length protein (22). It will be interesting to determine whether amino terminal fragments of huntingtin that may be produced by ubiquitin-mediated catabolism, or by caspases involved in apoptotic death (see below), have an increased interaction with GAPDH.

The interaction between GAPDH and the gene products from four different triplet expansion disorders suggests that that the fundamental biochemical defect in these diseases may be similar. The strong evidence for energy impairment in these neurodegenerative diseases (see below) makes the association with GAPDH particularly intriguing. Neurons are exquisitely sensitive to reductions in ATP generation and the direct interaction of these gene products with GAPDH may be the initial step in the impairment of glycolysis. To this end, it will be interesting to determine if neurons derived from HD brains have a reduced ability to synthesize ATP and to determine if this defect can be overcome by bypassing the GAPDH requirement in glycolysis by using pyruvate as an energy source.

The widespread distribution of GAPDH does not help explain the regional specificity of neuropathology in any of these diseases. However, because access of GAPDH polyglutamine tracts may be limited by other interacting proteins, it is possible to envision selectivity of a GAPDH interaction by indirect mechansims. Such cells may then be impaired in their ability to carry out glycolysis or to perform other functions central to energy metabolism. The resulting energy impairment may then initiate a degenerative cascade that results in eventual apoptosis and loss of particular neuronal cells in the characteristic pattern of each disease.

The toxic fragment hypothesis

There is a growing body of evidence that polyglutamine tracts on their own and that specific fragments of these novel proteins may be toxic to cells. For example, polyglutamine toxicity has been demonstrated in cell culture and in mice transgenic for polyglutamine-containing proteins. The evidence supporting toxicity of proteins or protein fragments containing polyglutamine tracts is summarized below.

Targeted disruption of the murine HD gene

Toxicity of a huntingtin fragment was raised by one of the three groups that generated targeted disruption of the murine HD gene, Hdh (131). Although each group show that homozygous disruption of huntingtin results in embryonic lethality (45, 131, 186) , our group reported phenotypic differences in heterozygous animals. Animals heterozygous for a disruption in the huntingtin promoter show no behavioral or neuropathological defects (186). In contrast, animals heterozygous for a targeted disruption in exon 5 of Hdh exhibit impaired performance on neurobehavioural tests (131). Detailed morphometic analyses demonstrated that these animals have significant and specific neuronal loss in the subthalamic nucleus and globus pallidus (131). Interestingly, electron micrographs of brain tissue obtained from these animals show neurons with the unmistakable hallmarks of apoptosis not seen in controls (Figure 2). The absence of any phenotype in heterozygous animals with promoter mutations and no possible protein product compared to animals with a targeted mutation in exon 5 and a phenotype suggests that this postulated truncated fragment could be toxic to specific cells.

Although the mice homozygous for a targeteddisruption in the Hdh exon 4 would also be expected to produce a truncated huntingtin fragment, these mice were reported to have no significant neuropathological changes (45). These mice were not subjected to the detailed morphometric and behavioral analyses as were the exon 5 knockout mice, and it is possible that similar subtle deficits may not have been observed. Moreover, because the exon 4 and exon 5 knockout mice were gen-

erated in different genetic backgrounds (45, 131), it is also possible that additional differences between the two strains may account for the phenotypic differences between these animals. Alternatively, there may be true differences between these animals reflecting differences in the effect of truncated fragments of different sizes.

Mice transgenic for huntingtin exon 1

A recent study has described a complex progressive neurological phenotype in mice transgenic for the human huntingtin promoter and first exon with a CAG repeat size of approximately 130 (111). Expression of this transgene produces a very truncated huntingtin fragment ending just downstream of the polyglutamine repeats. A germ line chimeric founder with five apparent integration sites was produced upon microinjection of this transgene. Segregation of these integrations generated lines that displayed a complex phenotype that includes motor aspects such as clutching of the feet when picked up by the tail, tremor, handling-induced epileptic seizures, and involuntary stroking of the nose and face and of kicking or scratching with the hind limbs. Shaking of the trunk and a consistent loss of balance when turning, sitting on hind limbs, or reaching around to groom their backs is also observed (111). Other features of the phenotype include weight loss despite food intake, characteristic vocalizations, frequent urination, and atrophied or vestigial reproductive systems that result in female sterility and reduced male fertility (111). The mice die suddenly of an undetermined cause.

Gene dosage correlates with progression of the phenotype, as animals with multiple transgenes show an earlier onset, a more severe clinical course, and an earlier death than animals with a single transgene (111). RT-PCR studies show that the transgene, when expressed, is observed in all tissues at levels ranging from 31-77% of the endogenous level. Western blot analysis confirms protein expression in brain and heart.

Although the mice display a neurological phenotype, analysis of neuropathology in these animals show little similarity to that observed in HD patients. There is no obvious morphological evidence of neurodegeneration in the brains of these animal with normal neuronal density and morphology observed in the striatum, cerebellum, and spinal cord (111). Interestingly, Mangiarini et al. (111) report that the brains of the transgenic animals are approximately 19% smaller than control littermates. However, because the transgenic animals weigh approximately 60-70% less than normal littermates at the end stages of the disease, it is not clear whether the decrease in brain weight represents shrinkage of brain tissue due to weight loss or specific neuronal loss.

Although the motoric symptoms of these transgenic mice may be characteristic of a basal ganglia defect, these mice have problems that are not found in HD such as atrophy of reproductive organs. Nevertheless, these results clearly show that expression of a truncated fragment of huntingtin carrying a highly expanded polyglutamine tract is sufficient to result in a progressive neurological phenotype.

Polyglutamine toxicity in MJD

In studies designed to probe the molecular consequences of expanded polyglutamine tracts in Machado-Joseph disease (MJD), Ikeda et al. (83) cotransfected a wide range of different sized MJD proteins with β-galactosidase or luciferase reporter plasmid in COS cells. They observe that wild-type MJD proteins with polyglutamine repeats in the normal range (26 and 35) does not appear to cause obvious differences in morphology or numbers of cells (83). However, the reporter plasmid is selectively lost in cells transfected with MJD alleles with 79 polyglutamines (MJD79), suggesting that the cotransfected cells were dying (83). Comparison of the protein profiles by Western analysis show that lysates of cells transfected with MJD79 have high molecular weight smears resistant to boiling in SDS, suggesting that this protein becomes covalently modified and forms insoluble precipitates in dying cells (83). Immunocytochemistry experiments show that while cells transfected with MJD alleles with shorter CAG lengths have uniform cytoplasmic staining, cells transfected with MJD79 show punctate staining patterns that appear to correlate morphologically with dying cells.

Ikeda et al. (83) constructed plasmids that express very truncated MJD proteins essentially consisting of extended polyglutamine tracts, or extended polyglutamine tracts followed by 42 amino acid residues derived from the carboxy-terminal portion of the MJD gene product. When transfected into COS cells, these truncated constructs with 35 glutamines have no apparent proapoptotic effect, while the constructs with 79 glutamines result in clear apoptosis as observed by condensed nuclei and DNA fragmentation (83). The apoptotic effect is dominant, modulated by gene dosage, and requires translation into polyglutamine, as insertion of the CAG tract into the 3' UTR of the expression plasmid abrogated the apoptotic potential (83).

Toxicity of truncated MJD constructs with long polyglutamine tracts is also evident in vivo when

assayed under the control of the Purkinje cell-specific L7 promoter (83, also see chapter by Burright et al., this issue). In these experiments, transgenic mice containing a truncated MJD allele with 79 CAG repeats display a clear gait disturbance and ataxic wide-based hindlimb posture as well as severe cerebellar atrophy (83). In contrast, neither a full length MJD allele with 79 CAG repeats or a truncated MJD allele with 35 CAG repeats resulted in a phenotype in transgenic mice (83). The combined in vitro and in vivo data support the hypothesis that protein fragments containing expanded polyglutamine tracts result in cell death.

Why are the truncated proteins more effective in causing cell death? If toxicity is dependent on exposure of the polyglutamine tract, folding of the flanking residues in the full length protein may limit exposure of the polyglutamine tract to other proteins in the cellular milieu. Alternatively, cells may contain factors that lead to or regulate the generation of cleavage fragments that result in exposure of the polyglutamine tract. Because these proteins may be cell-type specific, cell specific cleavage may help to explain the regional selectivity of cell loss in each of the polyglutamine expansion diseases.

Neurodegeneration in SCA1 transgenic mice

The gene responsible for neurodegeneration in spinocerebellar ataxia type 1 (SCA1) is ataxin-1 (4). Like other proteins involved in trinucleotide repeat diseases, ataxin-1 is a widely expressed cytoplasmic protein (153). However, in Purkinje cells, which degenerate in SCA1, ataxin-1 is found in the nucleus as well as the cytoplasm (153).

Transgenic mice were created that specifically express human SCA1 cDNAs with either 30 or 82 CAG repeats to Purkinje cells (23, also see Burright et al., this issue). No neurological phenotype was observed in animals transgenic for SCA1-30. However, five lines transgenic for SCA1-80 displayed a prominent ataxic phenotype and distinct Purkinje cell loss (23). Interestingly, Western blots failed to detect intact SCA1-80 protein in total brain extracts prepared from transgenic animals despite the presence of plentiful transgenic mRNA in these animals and positive results by immunohistochemistry using the same antibody (23).

In comparison to the HD and MJD transgenic animals, the SCA1 transgenic animals displayed a neurological phenotype with expanded CAG tracts within the context of the full length protein. However, failure to detect full length transgenic ataxin-1 in this study is intriguing. It will be interesting to determine if ataxin-1 is processed to smaller fragments in these animals and whether it is the presence of these smaller fragments that leads to neurodegeneration.

Polyglutamine toxicity in Escherichia coli

Interestingly, GST-polyglutamine fusion proteins have been found to be toxic in E. coli. Onodera et al. (136) generated a series of GST-fusion proteins containing varying lengths of polyglutamine tracts derived from DRPLA. While expression of GST-fusion proteins containing 10-35 glutamines were well tolerated by E. coli, expression of GST-fusion proteins with $\geq$ 59 glutamines markedly inhibited their rate of growth. Colony forming assays demonstrated that the reduction in growth rate is caused by an inability to replicate, which is synonymous with cell death in prokaryotes. The toxicity is specific to polyglutamine, since frameshifted versions of the GST-fusion proteins that encode polyalanine tracts do not inhibit bacterial growth. Additionally, toxicity is not due to glutamine depletion since glutamine supplementation failed to rescue the cells.

While postmitotic neurons and rapidly dividing E. coli cells are clearly very different, the observation that polyglutamine tracts are toxic in simpler systems opens many possibilities for further studies. Model systems such as bacteria or yeast will likely be very useful in characterizing candidate pathways of polyglutamine-mediated toxicity and for testing the extent to which glutamine repeats act as polar zippers that modify protein-protein interactions (139).

Apoptosis in HD

Although the precise biochemical mechanisms underlying cell loss in HD is not yet completely understood, the available evidence supports an apoptotic, rather than necrotic, mode of cell death. The lack of an inflammatory infiltration in HD brains argues against necrotic cell death (71). Animals heterozygous for a disruption in exon 5 of huntingtin have obviously apoptotic neurons. Huntingtin itself has been shown to be an apoptotic substrate (62). HD brains show increased levels of DNA strand breaks typical of apoptotic cells as measured by TUNEL (Terminal transferase-mediated deoxyuridine triphosphate nick end labeling) assays (41, 141, 171). Finally, oxidative and excitotoxic stresses, both of which are known inducers of apoptotic death, have been implicated in HD (7). However, because each of these observations is subject to methodological limitations, it should be noted that CAG expansion in huntingtin has not yet been shown to result unequivo-

cally in apoptotic cell death. Such a demonstration will require the production of bona fide cell culture and animal models of HD. The major challenges in current research are to define the molecular pathways leading to cell loss in HD as a result of CAG expansion and to use this knowledge to devise novel strategies for therapeutic intervention.

Apoptotic machinery and HD

Caspases: Caspases (cysteinyl aspartic acid-specific proteases) are a family of proteases related to CED3, the product of a gene required for programmed cell death in C. elegans (46, 113, 184). To date, 11 mammalian caspases have been identified and are classified into families based on their sequence homology, function, and substrate specificity (Figure 3). Several caspases, most of which have long prodomains, are thought to act as upstream activators by relaying apoptotic signals from the plasma membrane, or elsewhere in the cell, to downstream effector caspases that have shorter prodomains. Those capsases with demonstrated or potential regulatory roles include caspases 2 (ICH-1) (181), 6 (Mch2) (53), 8 (FLICE, MACH, Mch5) (14, 124), 9 (ICE-LAP6, Mch6) (43, 164),10a (Mch4) (52), and10b (FLICE-2) (174). The effector family, which includes caspases 3 (apopain, Yama, CPP32) (53, 133, 170) and 7 (Mch3, ICE-LAP3, CMH-1) (42, 55, 186), is considered to have direct roles in the dismantling or inactivating of crucial cell constituents. A third caspase family is involved in cytokine maturation as well as apoptosis. This family includes caspase 1 (ICE) (26, 184), caspase 4 (TX, ICH-2, ICE relII) (51, 88, 122), and caspase 5 (ICE relIII, TY) (50, 122).

Caspase 3 has been shown to be directly responsible for the proteolytic cleavage and inactivation of key homeostatic nuclear proteins during apoptosis (54, 133, 170). These targets include poly(ADP-ribose) polymerase (PARP), an enzyme involved in genome surveillance and DNA repair in stressed cells (133, 170), and the 460 kDa catalytic subunit of DNA-dependent protein kinase (DNA-PK), an enzyme essential for DNA double-strand break repair (25). Additional nuclear cas-

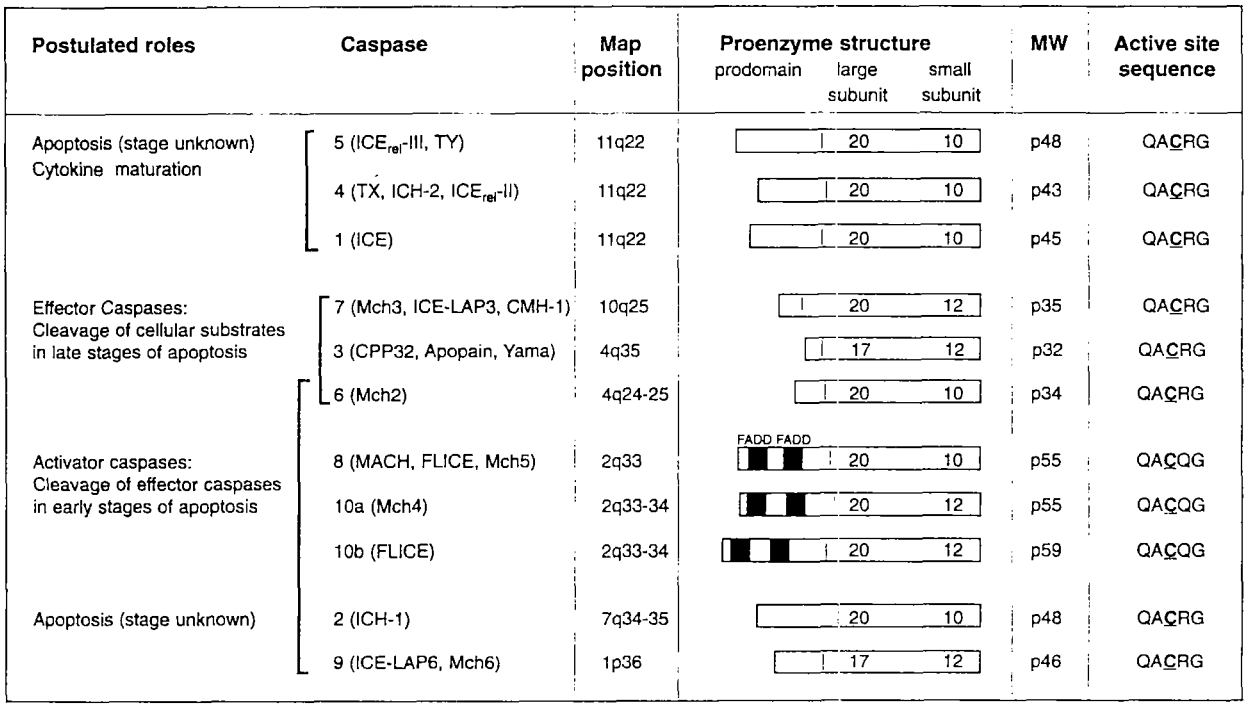

Figure 3. Representation of the caspase family of apoptotic proteases. The 11 known caspases are grouped into subfamilies based on sequence homology, substrate specificity, and known roles in apoptosis. For each protease, the caspase nomenclature and previous names are given. The chromosomal map position for human genes are noted. Zymogen forms of the proteases are shown diagrammatically, with prodomains, large subunits and small subunits marked. FADD-homologous domains present in the prodomains of caspases 8, 10a and 10b are shown in closed boxes. The molecular weight of the zymogen form is shown. Amino acids sequences surrounding the active site cysteine (underlined and bold) are also shown.

pase 3 substrates include the U1-70K small ribonucleoprotein and heteronuclear ribonucleoprotein C of the spliceosome complex (113). Caspase 3 also cleaves sterol regulatory element binding proteins (SREBPs) (178), and may cleave actin (161), fodrin (35, 66, 112), and Gas2 (18). Additional caspase substrates have been identified and shown to be cleaved during apoptosis, although the individual caspase family members responsible for their proteolysis have not been unambiguously identified. Because most (but not all) of these proteolytic "victims" are cleaved at caspase 3 consensus sites (DXXD), it is likely that this enzyme (or its functional counterparts, e.g. caspase 7) is responsible. All of this work substantiates an important role for caspase 3 as an effector of the cell death machinery.

The importance of caspase 3 as a crucial effector of neural apoptosis has been recently demonstrated by generating mice deficient in this enzyme (98). These mice are smaller than their normal or heterozygous littermates, die either in utero or within 3 weeks of age, and exhibit grossly abnormal neural structures that result from a profound inability to lose neurons appropriately in development (98). For example, the brains of these mice often protrude through secondary defects in the skull. The brains themselves also have ectopic cell masses with apparently normal composition and organization of neurons and glia (98). The extensive supernumerary cells in these masses result in increased indentations (98). These results strongly suggest that abolishing caspase 3 activity profoundly affects the ability to lose cells appropriately in the developing brain, a process that occurs by apoptosis. This was confirmed by showing that caspase 3-deficient animals had little or no evidence of apoptotic cells in their brains (98).

Mitochondria, oxidative stress and energy metabolism: Mitochondria and energy metabolism are known to play roles in HD and other neurodegenerative diseases including Parkinson's Disease, Alzheimer's Disease, cerebellar degenerations, and mitochondrial encephalopathies (7, 10, 33). For example, defects in mitochondrial electron transport have been demonstrated in HD patients (68, 84, 138). Additionally, proton nuclear magnetic resonance studies show that the occipital cortex and basal ganglia of symptomatic HD patients have increased lactate concentrations (91) indicative of a block in respiration. HD and MJD patients also have increased lactate-pyruvate ratios in cerebrospinal fluid, further supporting impaired oxidative phosphorylation as playing a role in these disorders (91,115).

Mitochondrial DNA is extremely sensitive to mutagenic damage, not only because it is in close proximity to the production of reactive oxygen species, but also because it lacks protective histones and many pathways of repair (176). Because mtDNA encodes very little nonfunctional DNA, accumulated mutations are expected to result in a progressive impairment in function with aging that is due to defects in the subunits of electron transport proteins encoded by mtDNA (17, 31, 32, 70, 116, 163, 167). These defects may result in small cumulative changes in energy metabolism that may exert deleterious effects only later in life when the susceptible cell falls below a threshold in energy production (38, 70).

Mitochondrial dysfunction may be particularly relevant to neurodegenerative diseases because damaged mitochondria would be expected to have particularly deleterious effects in nonproliferating neural cells. Interestingly, mitochondria are recycled in non-dividing cells such as neurons, where production of new mitochondria is balanced with the degradation of damaged mitochondria that are recognized as dysfunctional based on accumulated lipid peroxidation (67, 118). Because impaired mitochondria accumulate free radical damage to their membranes at a lower rate than normal mitochondria, they may be recycled less frequently than their normal counterparts (38). The eventual population of impaired mitochondria within the cell has been suggested to lead to further reductions in respiratory capability, particularly in aging cells (38). This mechanism may be particularly relevant in late-onset diseases such as HD.

There is a substantial body of literature supporting oxidative stress as being involved in HD. For example, chronic administration of 3-NPA, a neurotoxin derived from plants and fungi, results in lesions in the caudate and putamen of primates that leads to spontaneous dystonia, dyskinesia, and cognitive deficits (21, 48, 49, 63, 64, 107). 3-NPA is an irreversible inhibitor of succinate dehydrogenase (complex II) in mitochondria (30). This agent has been shown to cause apoptosis in cultured striatal and cortical neurons (12). In 3-NPA treated animals, neurons that stain positive for NADPH diaphorase are spared whereas spiny striatal neurons are depleted and show proliferative changes in dendrites similar to changes in HD (8, 16, 20, 21, 63, 64, 137). There is a progressive locomotor deterioration beginning with a hyperactive phase with no obvious striatal pathology that later resolves into a hypoactive phase with striatal pathology (15, 16, 20, 63, 95, 137). Finally, 3-NPA toxicity is dosage and age-dependent (8, 15, 21, 95). Taken together, these results suggest that 3-NPA treated ani-

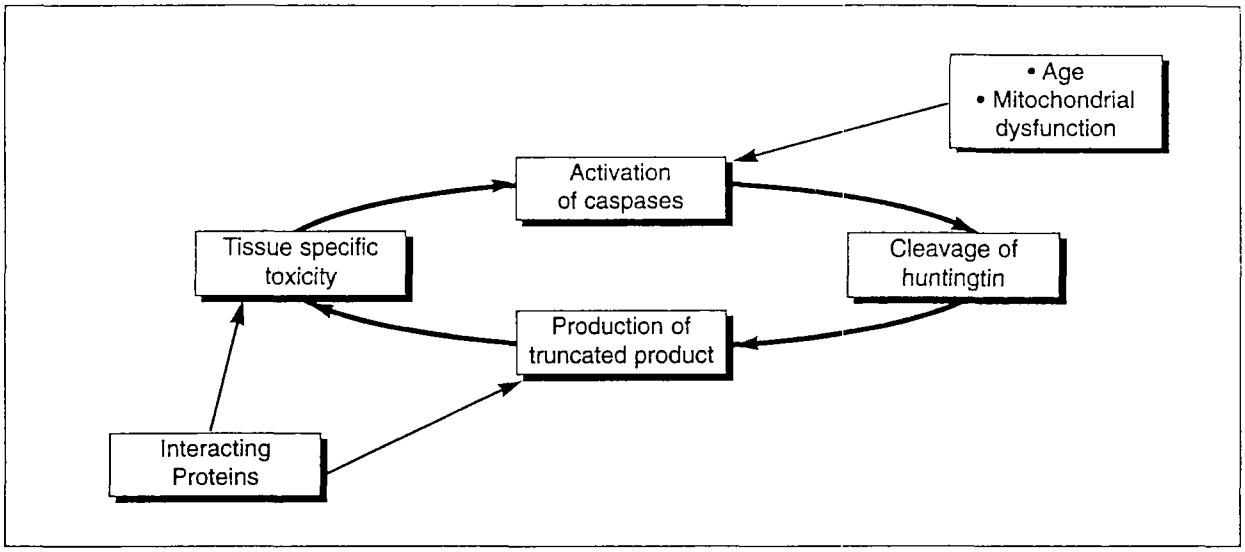

Figure 4. Positive feedback loop model illustrating how pathogenesis in HD may be initiated. Contributions from aging cells, mitochondrial function, and interacting proteins are noted.

mals develop many of the clinical and pathological features of HD.

Impairments in mitochondrial energy metabolism may lead to a secondary excitotoxic state due to opening of NMDA receptor channels by interfering with ion pumps that act to maintain the potential across neural membranes (10). Because much energy is required to fully repolarize synaptic membranes after a depolarizing stimulus, impairments in ATP production may lead to prolonged or subthreshold opening of voltage-gated dependent Ca2+ channels or by decreasing the voltage-dependent protective Mg2+ block of NMDA channels (47, 181). As a result, cells with mitochondrial defects may have NMDA channels that can be activated by endogenous levels of glutamate, resulting in secondary excitotoxic death. Supporting this hypothesis is the observation that excitatory amino acids can block degeneration of cortical explants derived from 3-NPA-treated animals (108).

The ability of mitochondrial perturbations to result in HD-like lesions is particularly intriguing with respect to the prominent role mitochondria are now thought to play in the regulation of apoptosis. A growing body of evidence suggests that mitochondria may be the cellular site at which the decision to commit irreversibly to death is made, primarily through mechanisms that involve the bcl-2 family of proteins. Many members of the bcl-2 family are integral membrane proteins found in intracellular membranes such as the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope (49, 69, 92, 93, 143, 183). The extensive bcl-2 family can be divided into two protein classes that exhibit proapoptotic or antiapoptotic properties (49, 69, 92, 93, 143, 183). The balance between the pro- and anti-apoptotic members of the bcl-2 family determines whether the cell will live or die (49, 69, 92, 93).

Three recently described functions of the bcl-2 family members occur at the outer mitochondrial membrane. For example, the mitochondrial membrane has been shown to serve as the site of interactions between anti-apoptotic bcl-2 members, CED4 (a gene product from C. elegans that is essential for apoptosis) or its as yet unidentified mammalian equivalent, and the activator caspases (28, 182). It has been suggested that these interactions serve to sequester activator caspases to the mitochondria, thus preventing the ability of these caspases to respond to apoptotic signals originating at the plasma membrane (28, 182). A second mitochondrial function of bcl-2 is in the regulation of cytochrome c localization. Cytochrome c is normally found between the inner and outer mitochondrial membranes where it serves to transfer electrons from complex II to cytochrome oxidase. Interestingly, cytochrome c is released from mitochondria as a very early event in apoptosis and is required for activation of effector caspases by mechanisms that are not yet known (88b, 183b, 105b, 131b, 113b, 32b). The third intriguing mitochondrial function newly ascribed to members of the bcl-2 family results from the observation that one member of this family, Bcl-xL, has been shown to form an ion

channel in synthetic lipid membranes (120). If such pores are also formed in mitochondrial membranes, they may contribute to the collapse of the mitochondrial membrane potential that occurs at some point during apoptosis (140, 154, 158, 158, 185).

The roles of huntingtin in apoptosis

Goldberg et al. (62) have recently demonstrated that huntingtin is also specifically cleaved by caspase 3 and that the extent of cleavage is modified by increasing polyglutamine lengths. Cleavage of huntingtin has also been demonstrated using apoptotic extracts prepared from chicken hepatoma cells committed to apoptosis (62). In these experiments, incubation of in vitro translated huntingtin with purified caspase 3 or apoptotic extract results in cleavage of the radiolabelled substrate, while huntingtin remains intact when exposed to non-apoptotic extract or buffer-only conditions. In control experiments, cleavage of PARP is also observed only in apoptotic extracts or in the presence of caspase 3. Furthermore, endogenous huntingtin is cleaved during apoptosis in cultured cells (130).

The identity of the caspase in these apoptotic extracts was shown by inhibitor studies to be one of the apopain family members. Proteolytic activities of the different protease families can be distinguished by inhibitor profiles using selective tetrapeptide aldehydes designed to mimic the substrate P4-P1 amino acids at the site of cleavage. For example, Ac-DEVD-CHO specifically inhibits members of the apopain family of caspases (Ki < 1 nM) whereas Ac-YVAD-CHO is a potent inhibitor of the ICE-like caspases (Ki = 12 mM) (133). Cleavage of huntingtin is inhibited only with Ac-DEVD-CHO. In contrast, Ac-YVAD-CHO or CrmA, a baculovirus protein that specifically inhibits ICE-like caspases, are both ineffective at inhibiting huntingtin cleavage (62). Together these results establish the apopain family of caspases as being the enzymes responsible for huntingtin cleavage.

Purified recombinant caspase 3 was then shown to be an effective protease against in vitro translated huntingtin. Apopain cleavage results in a fragment of approximately 80 kDa that is recognized by an antibody raised again the amino terminus of huntingtin. Kinetic analysis showed that huntingtin is a relevant substrate for caspase 3, as the efficiency of huntingtin cleavage is comparable to the efficiency of PARP cleavage over the same enzyme concentration range (62). In addition, huntingtin alleles with expanded polyglutamine tracts appear to be subtly better substrates for caspase 3 (62).

Although the specific cleavage site was not mapped in this study, there is a cluster of DXXD sites, the consensus cleavage site for caspase 3 (133), confined to a narrow region within the amino terminus. Cleavage at one of these sites would be expected to produce a protein with a molecular mass of between 50-60 kDa. However, as the predicted truncated fragment contains the polyglutamine stretch, it would be expected that this protein fragment would run at a higher molecular weight on SDS-PAGE.

In transient transfection experiments, specific huntingtin breakdown products are observed to be the same size as the cleavage products generated in vitro by purified caspase 3 (62). A COS cell line stably expressing a truncated huntingtin allele was also prepared. Treatment of this stable line with camptothecin, a topoisomerase I inhibitor known to induce apoptosis, results in production of an appropriately sized huntingtin cleavage product coincident with caspase 3 maturation and PARP cleavage (62). These results show that huntingtin is cleaved in cells undergoing apoptosis. Further studies are necessary to determine whether cleavage of huntingtin occurs in vivo in humans. The development of appropriate animals models of HD will clearly be an important resource for these experiments.

A crucial question is whether caspase 3 cleavage of huntingtin is a cause or an effect of neurodegeneration in HD. How can cleavage of huntingtin in cells already committed to apoptosis be involved in the initiation of the neuropathology? One model explaining how huntingtin may be both a caspase substrate and an initiator of apoptosis postulates that huntingtin is involved in a positive feedback loop that results in apoptosis (Figure 4). For example, this model suggests that there is a basal level of caspase activity that may be related to the degree of stress in the cell, perhaps through an age-dependent decline in mitochondrial function. Although this basal caspase activity would be unable to trigger full-blown apoptosis, it may be sufficient to cleave small amounts of substrate. In particular, huntingtin with expanded CAG tracts may be especially vulnerable to cleavage by basal levels of activated caspases. If the cleavage products with expanded polyglutamine tracts are potentially toxic, as has been suggested for huntingtin, even basal levels of cleavage may initiate a positive feedback loop by adding additional stress to the cell, resulting in additional caspase activation, additional cleavage, and eventual apoptosis.

Several variations of this model can also be considered in which initiation of the feedback loop is due to different causes. In a kinetic argument, for example, huntingtin alleles with increasing CAG lengths may be

more susceptible to cleavage by basal levels of activated caspase 3 and thus more easily generate toxic levels of the amino-terminal fragment. Alternatively, toxicity of the amino-terminal fragment of huntingtin may be evident especially with polyglutamine expansion. It is also possible that normal turnover of huntingtin by proteases, such as those involved in the ubiquitin pathway, may generate huntingtin fragments with toxic potential. Additionally, age-dependent mitochondrial dysfunction may contribute to initiation of the feedback loop. Distinguishing among these hypotheses will require an understanding of the signal transduction pathways leading to caspase activation and cleavage of critical cellular substrates, including huntingtin, in apoptotic cells.

If the hypothesis that huntingtin alleles with expanded polyglutamine tracts may contribute to the initiation of apoptotic death in striatal neurons is validated, several novel therapeutic approaches can be envisioned. For example, agents designed to block caspase activation may be developed and tested for their efficacy in preventing neuronal cell loss in HD patients (132). One potential caveat to this line of potential treatments may be that although the neurons may not die, they may remain under considerable stress and as such their functioning may not be completely normal. A second route to novel therapies may make use of knowledge gained from the study of huntingtin and its interacting proteins by counteracting the biochemical effects of CAG expansion.

There is still much to learn about how huntingtin with expanded polyglutamine tracts result in loss of particular neurons in the brain. One major area of investigation will focus on common pathways leading to cell death for diseases associated with polyglutamine expansion. We need to understand the biochemical ways in which long polyglutamine tracts lead to cell death by developing sensitive systems with which to probe the fundamental molecular consequences of polyglutamine expansion. Superimposed on these mechanistic questions is the need to understand the distinct patterns of regional specificity of neurodegeneration for each disease. Experiments to address these questions will rely heavily on technologies using in vitro systems as well as transgenic and knockout animals for each member of this fascinating group of diseases.

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