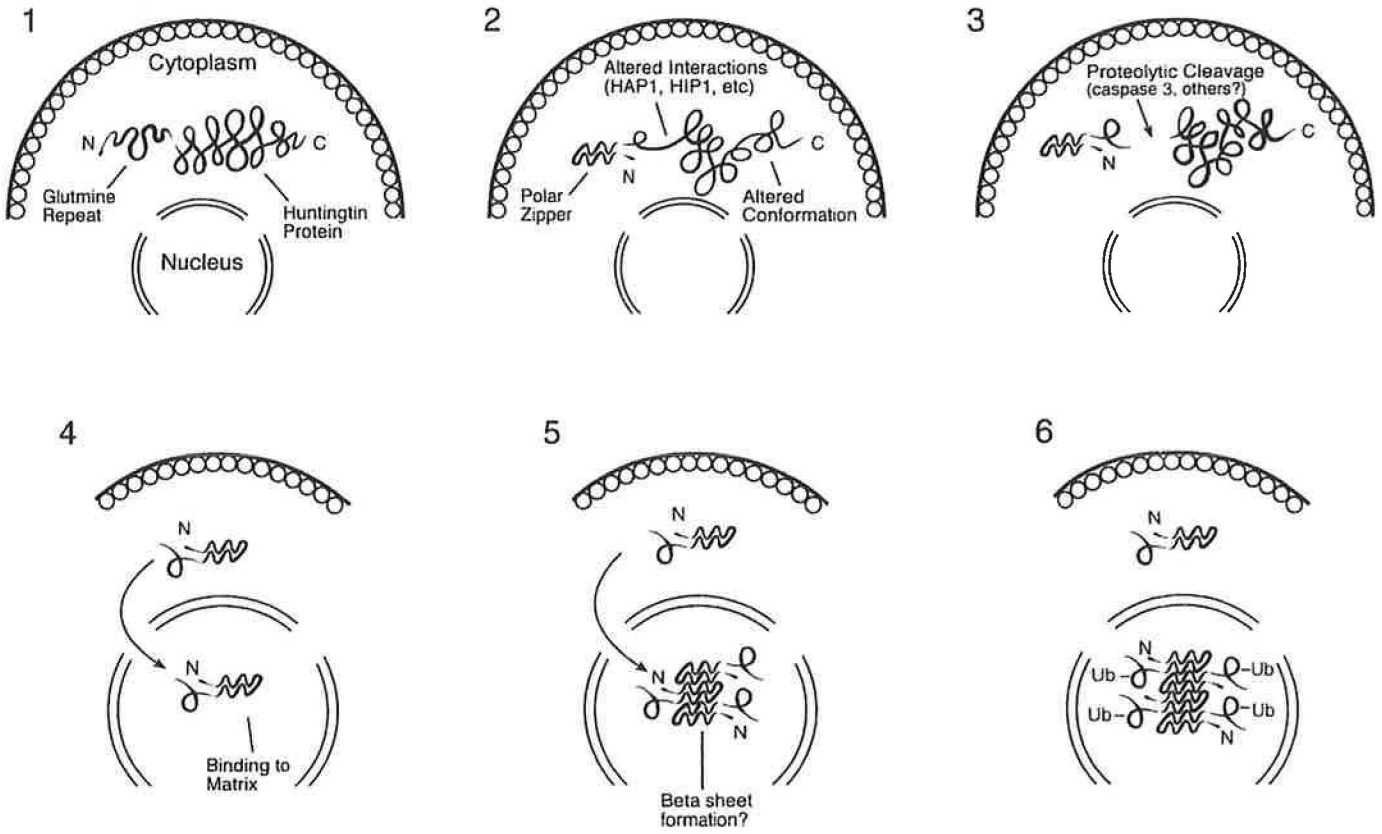
Polyglutamines & Neurodegenerative Diseases



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Internal Medicine Grand Rounds
University of Texas Southwestern Medical Center at Dallas

April 29, 2004

This is to acknowledge that Rody Cox, M.D. has not disclosed any financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Cox will not be discussing off-label uses in his presentation.

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Areas of Interest
Medical Education
General Internal Medicine
Human Biochemical Genetics
History of Medicine

I thank Jan Zerwekh and Heather Stieglitz, Ph.D. for their outstanding coordination and preparation of this presentation.

I also thank Ilya Bezprozvanny, Ph.D. of the Department of Physiology for sharing his important new studies on calcium homeostasis and for illustrations of Huntington's disease.

PATIENT HISTORY

R.J. was a 42 year old internist with a large and successful practice. He noticed that he was not as "sharp" and decisive as previously. He also occasionally dropped small objects which was unusual for him. When he was 45, his daughter asked him why his hands and feet occasionally twitched. R.J. was unaware of these rare involuntary movements and was not particularly concerned as these symptoms had occurred gradually.

Later that year, several of his long-time patients remarked that he seemed uncharacteristically impatient and irritable. The physician noticed that he could not control the involuntary twitching of his hands and feet. Moreover, he began to trip over uneven flooring. A year later, his gait was unsteady and he was clumsy. He, at the urging of his family, consulted a neurologist friend.

Family history revealed that he was estranged from his biologic father who had separated from R.J.'s mother when he was two years old. He knew his father had been institutionalized for a presumed psychiatric condition. He also had a paternal aunt who died in a psychiatric institution. On the basis of the physical examination, which I will describe later, a tentative diagnosis of Huntington's Disease (HD) was made.

INTRODUCTION

We will be comparing mutations in exons with those in introns during this presentation. Therefore, a brief review of gene structure, transcription and translation may be useful. A gene consists of a series of nucleotides arranged linearly. The gene is composed of regions called exons and introns. Exons comprise the nucleotides that determine the amino acid sequence of the proteins, that is, they are transcribed into mRNA which is then translated into the protein. Introns are regions of the DNA interspersed between exons. They are removed from the nuclear RNA by a splicing mechanism and therefore are not represented in the mRNA and cannot be translated into protein. The genetic code is a triplet of nucleotides, each triplet encoding an amino acid. CAG is the triplet for glutamine. It is the expansion of repetitive CAG triplets in DNA that will comprise our discussion of polyglutamines and neurodegenerative diseases today.

First, I would like to describe a unique mechanism of mutation which is responsible for more than a dozen different neurodegenerative diseases. The expansion of unstable trinucleotide in human DNA has been found to cause 15 neurological disorders, 12 are neurodegenerative diseases which are the results of expansion of the trinucleotide CAG repeats coding for polyglutamine tracts in specific proteins.

The expansion of trinucleotide repeats as a cause of human disease may be divided into two discreet categories. The first is when the triplet expansion occurs within introns and the expanded nucleotide triplets are <u>not</u> <u>translated</u> and therefore do not insert a series of an amino acid into the protein. Examples of these diseases are:

1) CGG expansion of chromosome Xq27.3 from the normal 6 to 52 triplets to the pathologic 200 to 1000 causes the Fragile X syndrome, an X-linked dominant. This is one of the most common causes of inherited mental retardation in males, as it is X linked. Patients

have a characteristic triad of mental retardation, a long and narrow facies with large ears and frontal prominence, and large testes (1).

2) CTG expansion at the myotonic dystrophy locus on chromosome 19q is the most frequent muscular dystrophy of adults and is an autosomal dominant. It is characterized by progressive muscle weakness and wasting together with myotonia – a delayed muscle relaxation and muscle stiffness. Cardiac muscle, CNS involvement with personality disorders, cognitive dysfunction and cataracts with distinctive multicolored subcortical lens opacities are associated findings(2).

3) GAA expansion in Friedreich's ataxia on chromosome 9q13-q21 is responsible for a progressive unremitting mixed cerebellar ataxia. This disease segregates as an autosomal recessive. It begins about puberty with clumsiness of gait and frequent falls due to truncal ataxia. The disease progresses to marked limb incoordination, impaired speech, severe neuropathy of motor and sensory fibers. Cardiac involvement, diabetes mellitus and skeletal abnormalities often accompany the neurological disease (3).

As described, these three diseases with trinucleotide expansions are not translated into protein and cause pleiotropic pathological and clinical manifestations. They also affect several different organ systems, although the diseases are primarily neurologic. These three non-translated triplet expansions will not be the subject of our discussion today.

Polyglutamine Expansions as a Unique Mutation in Exons

An important difference between the non-translated triplet expansion and the polyglutamine tract disease is that the fragile X, myotonic dystrophy, and Friedreich's ataxia can also be caused by a typical or classic mechanism of mutations such as chromosome deletions, translocations or inversions (1-6). The polyglutamine diseases can only be caused by expansion of CAG triplets in exons and are genetically distinct from diseases caused by classic mutations and by the following criteria.

1) They are not only dominants but they are dominants with a gain of function. That is, the polyglutamine expansions produce an abnormal phenotype in the presence of the normal allele. If, due to loss of heterozygosity, an individual has only one normal allele at that particular locus, they have a normal phenotype. This has been observed in several individuals with a loss of heterozygosity at the relevant locus as well as in transgenic animal models (7,8). Disease is dependent on an expansion of the polyglutamine tract for development of the disease phenotype.

2) Expansion of the polyglutamine tract in successive generations is associated with earlier onset of disease thus explaining the "anticipation phenomenon" in families where the disease is segregating (9).

3) Unstable CAG repeats cause polyglutamine associated diseases only at specific anatomical locations in the brain and affect specific types of neurons. The areas involved are characteristic for each of the neurodegenerative diseases. The implication is that the affected protein targets the neurons and regions involved. Polyglutamine repeats are present in many other eukaryotic proteins including several transcription factors – TATA

binding protein (TBP), a basic transcription factor has a streak of 38 uninterrupted glutamines(4,10). This glutamine repeat was believed to be stable from generation to generation. However, recently an expansion of these CAG triplets was associated with spinal cerebellar ataxia (SCA) type 17.

4) The length of expansion of the CAG triplets with the corresponding lengthening of polyglutamine tracts correlates with the severity of the disease in an individual.

5) The unstable CAG repeats therefore do not adhere strictly to the rules of Mendelian inheritance. They change size in successive generations in affected families. Usually expanding to produce earlier and more severe disease, but occasionally they may reduce the number of triplet repeats with shortening the polyglutamine tracts and thereby skipping a generation (penetration) or exhibiting reduced expression (milder or delayed disease). The expansion of the CAG triplets occurs predominantly during spermatogenesis (9a). Therefore, increase in the number of polyglutamines is inherited from the father. Maternal polyglutamine expansions are relatively stable or rarely may undergo a reduction in length.

6) In most families, normal size repeats, 6 to 35 polyglutamines, are transmitted stably despite their polymorphic nature, thus accounting for the rarity of these polyglutamine neurodegenerative diseases. The pathogenesis and pathophysiology of polyglutamine associated neurodegeneration is not completely known, but studies which I will describe later suggest that the pathogenesis of neuronal death has relevance to common neurodegenerative diseases such as Alzheimer's, Parkinsonism, amyotrophic lateral sclerosis and Prion diseases (10). Hence, the increased interest in polyglutamate neurodegeneration.

Description of Polyglutamine Neurodegenerative Diseases

I will briefly describe several representative polyglutamine hereditary neurodegenerative diseases as examples.

1) **Huntington's Disease**

Huntington's chorea was first described by George Huntington in 1872 and his description, published in the *Medical and Surgical Reporter*, Philadelphia, is one of the classic descriptions of the disease (4,11). Huntington's disease is one of the commoner neurodegenerative hereditary diseases due to expansion of polyglutamine tracts. The incidence of the disease varies from approximately 5-15 per 100,000 patients (12). The highest frequency of Huntington's disease in the world occurs in Venezuela at the edge of Lake Maracaido (13,14). The gene was introduced into this community sometime in the early 19th century and the gene frequency has increased rapidly as the result of the isolation and inbreeding of affected persons in this relatively isolated area. This population afforded the unique research opportunity for investigating the natural history of the disease as well as understanding the clinical features of the neurodegenerative disease.

Huntington's disease is a slowly progressive disorder of insidious onset (Fig. 1). It is difficult at times to gauge the exact age of onset since families who are aware that the disease is segregating in their kindred often either interpret any slight deviation from normal as the onset of the disease, or if they are in a state of denial, they may wait until the signs are relatively advanced before attributing this disease to the Huntington abnormality.

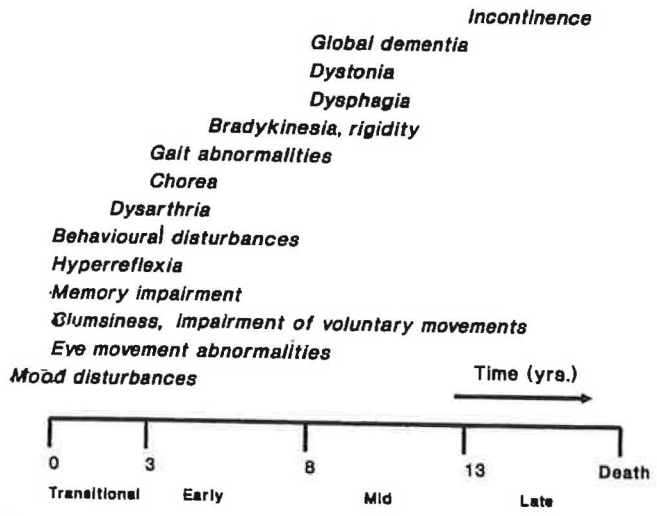


Fig. 1. Clinical phases and associated signs and symptoms in the natural history of Huntington's disease.

The first symptom is usually involuntary movements which develop insidiously (15). They are most conspicuous in the face and upper limbs and are usually more rapid and jerky than the movements of Sydenham's chorea. As the disorder progresses, it leads to dysarthria and ataxia of the upper limbs and abnormality of gait. Motor abnormalities are predominantly associated with the extrapyramidal motor system. Hypokinesia, rigidity and dystonia mark the later phases of disease progression. Choreic movements of the face are common and present with pouting, irregular grimacing, twitching of the face and chorea with protruding of the tongue. Late in the disease, bradykinesia and rigidity similar to the core features of Parkinson's disease predominate. This is observed in their gait. In the final stages of the illness, the patients will become severely rigid and grossly akinetic. Mental changes gradually develop, usually a few years after the onset of the involuntary movements. They consist of progressive dementia. Most patients become inert, apathetic and irritable. Delusions may occur and outbursts of excitement are not uncommon. Suicide is exceptional. The clinical picture does not always exhibit the classical features just described. Dementia may precede involuntary movements or the latter may never appear. The disease may occur in childhood. The youngest patient ever described was two years old when his disease started, whereas some patients were noted to first develop the signs of the disease in their mid-eighties. An explanation for this variation in onset will be discussed and involves the number of repetitive glutamines that occur in the huntingtin gene.

The HD gene located on chromosome 4p16.3 is large and encompasses 67 exons and spans over 200 kb (16). It is ubiquitously expressed as two transcripts, one 10.3 kb and the other 13.6 kb in length (17). They differ in the size of the 3'UTR. Structural analysis of the promoter gene is consistent with its being a housekeeping type gene; therefore, the mRNA and huntingtin protein are present in low concentrations in most tissues (18). The HD gene lacks homology to any previously characterized gene and encodes a protein of 3,144 amino acids with a predicted molecular mass of 348 kDa. The polyglutamine tract starts at residue 18 in exon 1 and is followed by a stretch of 29 consecutive prolines.

The mutation underlined HD is an expansion of the CAG polyglutamine tract in the first exon (16). The CAG repeat length is highly polymorphic in the population and a repeat size in the normal ranges from 6-35. In affected individuals, CAG expansion ranges from 36-121. Adult onset patients usually have an expansion from 40-55 CAG triplets, whereas juvenile onset patients have an expansion greater than 60 (9,20).

HD is distinguished by a very distinctive pathology as shown in Figure 2. The main diagnostic feature is a marked atrophy of the caudate nucleus and less marked gliosis of the putamen (21). The cells lost initially are the medium spiny neurons with their connections to the globus pallidus and substantia nigra.

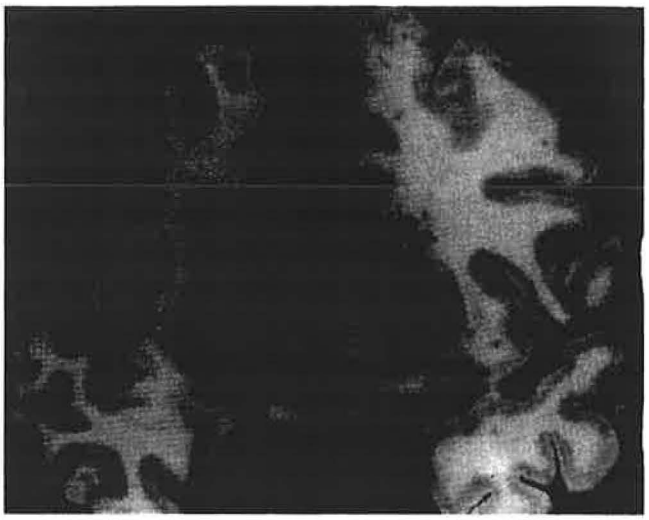


Fig. 2. Coronal brain sections. Severe striatal atrophy in the brain of a patient with HD (left) compared with a control (right).

Prior to the discovery of the HD gene, a definitive diagnosis of HD could be made in the presence of: (1) a positive family history consistent with autosomal dominant inheritance, (2) progressive motor disability involving both involuntary and voluntary movements, and (3) mental disturbance including cognitive decline, affective disturbance and other changes in personality. At present, diagnosis can be made with virtually 100% certainty when the clinical signs are consistent with HD and are confirmed by the demonstration of an expanded CAG repeat greater than 36 in the huntingtin gene (22).

In the past, it has been stated repeatedly that HD is completely penetrant. However, it is now clear that only a proportion of patients with CAG expansions between 36 and 41 may manifest the disease within their expected life span (22). As the number of CAG repeats is close to 36, the onset of disease might not be manifested.

Homozygosity for the HD gene is relatively rare as it occurs in the offspring of two parents affected with HD. However, in Venezuelan families, four of fourteen siblings were shown by DNA segregation analysis to be homozygous for the DNA haplotype with which the huntingtin gene was segregating (23). It is of great interest that the homozygous persons were indistinguishable from heterozygotes in the same family. This finding confirms the earlier conclusions that loss of heterozygosity for the HD gene where only one HD allele is present, the phenotype of those individuals was completely normal.

Therefore, the homozygous form of HD is different from other autosomal dominant inherited disorders such as familial hypercholesterolemia where homozygotes have a much more severe manifestation of the illness. In other trinucleotide expansion diseases such as

spinocerebellar atrophy (SCA), the homozygote may be more seriously affected than the heterozygote (24).

2) Spinocerebellar Ataxias (SCA)

The dominantly inherited spinocerebellar ataxias (SCAs) are a heterogeneous group of neurologic disorders characterized by a variable degree of degeneration of the cerebellum, the spinocerebellar tracts and brainstem neurons (Table 1) (25). The inter- and intra-familial variability of clinical pathological findings hampers the clinical classification of

TABLE 1 Molecular characteristics of polyglutamine neurodegenerative diseases

Disease	Gene locus	Gene product	Normal CAG(n)	Expanded CAG(n)	Protein localization	Special features	Brain regions most affected
SBMA	Xq11-12	Androgen receptor	9–36	38–62	Nuclear and cytoplasmic		Anterior horn and bulbar neurons, dorsal root ganglia
HD	4p16.3	Huntingtin	6–34	36–121	Cytoplasmic	Intermediate alleles: 29–35	Striatum, cerebral cortex
SCA1	6p22-23	Ataxin-1	6–44	39–82	Nuclear in neurons	Normal alleles >21 repeats interrupted with 1–4 CAT units	Cerebellar Purkinje cells, dentate nucleus; brainstem
SCA2	12q23-24	Ataxin-2	15–31	36–63	Cytoplasmic	Normal alleles interrupted with 1–2 CAA units	Cerebellar Purkinje cells, brain stem, fronto-temporal lobes
SCA3	14q24.3-31	Ataxin-3	12–41	62–84	Cytoplasmic		Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord
SCA6	19p13	CACNA1A	4–18	21–33	Cell membrane		Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	3p12-p21.1	Ataxin-7	4–35	37–306	Nuclear	Intermediate alleles: 28–35	Cerebellum, brain stem, macula, visual cortex
DRPLA	12q	Atrophin-1	6–36	49–84	Cytoplasmic		Cerebellum, cerebral cortex, basal ganglia, Luys body

this group of diseases (26). In the 1990's and recently, the mutational loci have been mapped for eleven distinct SCAs. The clinical features of these diseases are quite common to all eleven and are shared by them. These features include ataxia, dysarthria and bulbar neuron dysfunction. Certain of the diseases tend to have more eye findings such as cranial nerve palsies while others have predominantly choreoathetosis and myoclonus. Spasticity and neuropathy are also common among certain of these diseases and permits a distinction at the clinical level between the eleven discrete genetic entities, but the overlap in these clinical findings makes a distinction at the phenotypic level very difficult (26,27,28). (See Table 1 which characterizes six of the more common SCAs).

The gene products mutated in most of the SCAs are ataxin-1, ataxin-2, ataxin-3, ataxin-7 and atrophin-1. All of these proteins are novel and of unknown function. The gene product mutated in SCA-6 is the α-1A voltage gaited calcium channel (29,30). The location of the proteins vary: ataxin 1 is nuclear in neurons, ataxin 2, 3, 7 and atrophin 1 are all cytoplasmic. The translation of mutant proteins with expanded polyglutamines is probably related to their cellular and subcellular distribution. The clinical characteristics of the SCA and the neurons affected supports the hypothesis that these differences in

phenotypes are mediated by the type of protein affected. Table 1 also indicates the regions of the brain that are most significantly affected by each of the polyglutamine diseases (31).

The dynamic nature of the mutations in the SCAs, that is, the expansion of unstable CAG trinucleotide repeats, explains the clinical features common to this group of diseases. These expansions are also responsible for genetic anticipation similar to HD, that is, the occurrence of the disease earlier in individuals that have the most expanded polyglutamine tracts (25). The intergenerational CAG repeat instability often leads to further expansions of the polyglutamine stretches, particularly when they are inherited from the father and leads to an earlier onset and more severe clinical course. In all of these diseases, there is an inverse correlation between the number of repeats and the age at which the disease occurs. However, the age of onset for certain expanded ranges varies depending on which mutant protein is involved, hence, the phenotypic variations in the SCAs.

Genetic Counseling

The physician or geneticist may encounter a healthy person who, because of their family history, is at risk for HD or SCA and who wants to know whether they have inherited this illness. It is important to recognize that the risk varies, taking into account the subject's age. For a person in early adult life with an affected parent, the risk is very close to fifty percent. However, by the time the person has reached the age of 60 that risk will have diminished significantly. Table 2 is based on data from life analysis. It should be noted that the risk is much greater if the father is involved than if the mother is the one who carries the abnormal gene (4,9a).

Table 2. Risk for a Healthy Subject at 50 percent Prior R of HD Carrying the HD Gene at Different Ages

Age (years)	Risk
20.0	49.6
22.5	49.3
25.0	49.0
27.5	48.4
30.0	47.6
32.5	46.6
35.0	45.5
37.5	44.2
40.0	42.5
42.5	40.3
45.0	37.8
47.5	34.8
50.0	31.5
52.5	27.8
55.0	24.8
57.5	22.1
60.0	18.7
62.5	15.2
65.0	12.8
67.5	10.8
70.0	6.2
72.5	4.6

Predictive testing for Huntington's Disease, or HD, is relatively easy since one can examine the huntingtin gene for expanded CAG trinucleotide tracts. The psychological impact on individuals undergoing this testing has been studied and emotional stress is formidable. In this vulnerable situation, severe adverse reactions have occurred, most common is profound clinical depression. In those persons where the tests are negative, they are relieved, but the devastation of those who test positive makes testing for this gene an unpopular decision (32,33,34). The demand for predictive testing will probably increase if therapy for the disease is developed.

Prenatal testing is also available, although the ethics of terminating a pregnancy in a fetus who is likely to develop the disease in his fifties or early sixties presents many dilemmas (35). There is increasing need to study the ethical situation and continuing need for a longitudinal investigation to examine the psychological and social effects of testing for the HD gene.

Pathophysiology of HD

Relationship between CAG repeats and parental sex

Although CAG triplets are expanded in patients with HD, it has become apparent that continuing expansion of the CAG repeats occur much more commonly in males than in females. Therefore, children inheriting their abnormal gene from their fathers may have an increased severity of involvement as well as earlier onset of disease. Those who inherit the abnormal huntingtin gene from their mothers generally have an onset and severity similar to their mothers, since CAG expansion is unusual in females (4,9a).

The basis of the CAG expansion is not known, but it is clear from the data presented that trinucleotide expansion arises much more frequently during spermatogenesis than it does during ovogenesis. In fact, in those rare instances where the CAG repeats are reduced in number almost always occurs in the maternal genome.

Morphological features in polyglutamine expansion disorders

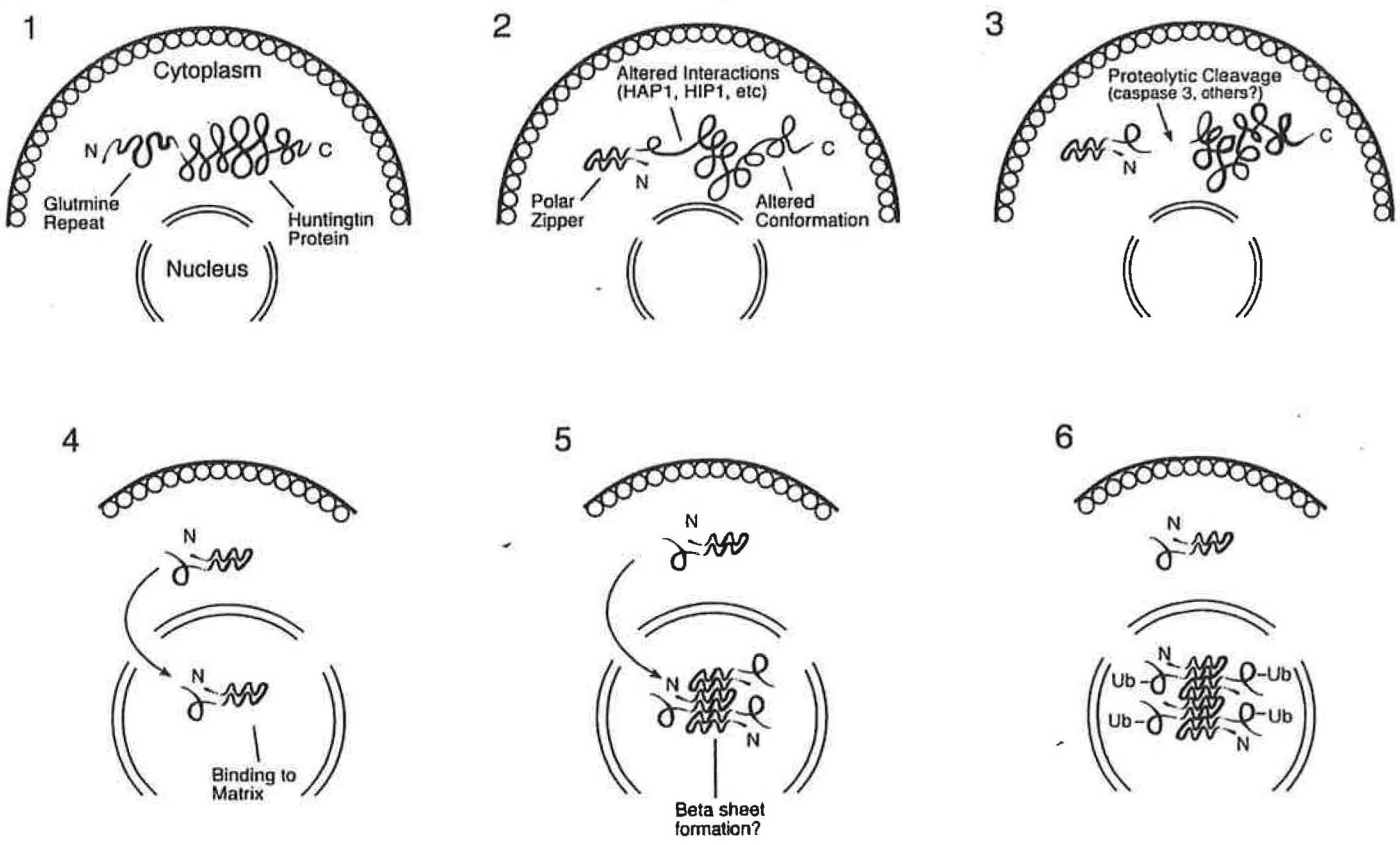
One reason for selecting the HD is that many of the studies indicate that HD and its mechanism of occurrence parallels and may be related to the more common diseases of neurodegeneration, Parkinson's disease, Alzheimer's disease and perhaps prion diseases. Therefore, the remaining presentation will stress the similarities in these relatively common neurodegenerative diseases and try to shed further light on the pathophysiology and mechanisms whereby neuronal death occurs (5,31,36-40).

Protein Misfolding

HD, spinal cerebellar ataxias, Parkinson's disease, Alzheimer's disease, and the prion diseases are all believed to share a common abnormality, which is misfolding of proteins. However, the mechanistic relationship between the misfolded proteins in these diseases and their neurotoxic effects remains unclear. We will explore several avenues that may be relevant to the neurodegeneration associated with the diseases noted above. The CAG triplet expansion present in HD occurs in the first exon of the huntingtin gene. Therefore, at the N-terminal, one has an expansion of polyglutamines (Fig. 3)(31). Normally there are 35 polyglutamines or less in this N-terminal region. However, in patients with HD, these expansions are significantly increased. The conformational state of the expanded polyglutamines is debatable and several hypotheses have been advanced. One is that the polyglutamines form a hairpin or polar zipper conformation which is an abnormality that tends to lead to aggregation (5,31,40-43). The polar zipper conformation of the polyglutamine stretch attracts an enzyme for proteolytic cleavage. Caspase-3 is a cysteine aspartate-specific protease and is responsible for cleaving the polyglumatine expansion from the huntingtin protein (44). The putative toxic protein fragments contain the polyglumatine tract and tends to form aggregates composed mainly of the truncated huntingtin polypeptides. These initially formed cytoplasmic inclusions are then later sequestered into the nucleus of the cell. There is evidence that the polyglutamine interactions between the truncated fragments attracts other proteins and these may contribute to neuronal dysfunction. Some of these proteins are transcription factors (45,46) and some of them are NADPH oxidases which are potent producers of peroxides and free radicals that may modify the proteins and lipids contained

in the neurons (47). Additional proteins detected in the neuronal inclusions are ubiquitin, the 19S and 20S proteasome complex and several of the molecular chaperones (48). Importantly,

Fig. 3



the components involved in protein folding and degradation are also associated with the intracellular inclusions in other neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and prion diseases which suggest that a common pathomechanistic principle may underlie these misfolding diseases in general (49).

Molecular Chaperones as Modulators of Polyglutamine Protein Aggregation (42,43,50)

Proteins are linear sequences of amino acids synthesized on ribosomes. This polypeptide chain must adopt the unique three-dimensional structure required for its function in a cell. Originally, it was believed that the folding process was autonomous in that it did not require additional factors or the input of energy. *In vitro* with simple proteins, for example ribonuclease A, the correct three-dimensional structure does form spontaneously as the newly synthesized protein leaves the ribosome. However, in the last 10 to 15 years, it has become clear that more complex 2 proteins or polypeptides, particularly if they contain hydrophobic regions of amino acids may misfold and aggregate forming intracellular inclusions that cannot be degraded.

Considering the amount of energy the cell has expended in synthesizing a protein, it is not surprising that a complicated machinery of proteins have evolved that assist other proteins to

fold. These proteins collectively named molecular chaperones associate with unfolded polypeptides preventing misfolding and aggregation. They promote the production or correct folding in an ATP-dependent manner. By this energy expenditure, the molecular chaperones inhibit unproductive interactions and allow the new proteins to fold more efficiently into its functional three-dimensional configuration as dictated by its amino acid sequence. In a sense, molecular chaperones are similar to human chaperones which prevent misguided or undesirable interactions between boys and girls (Fig. 4).

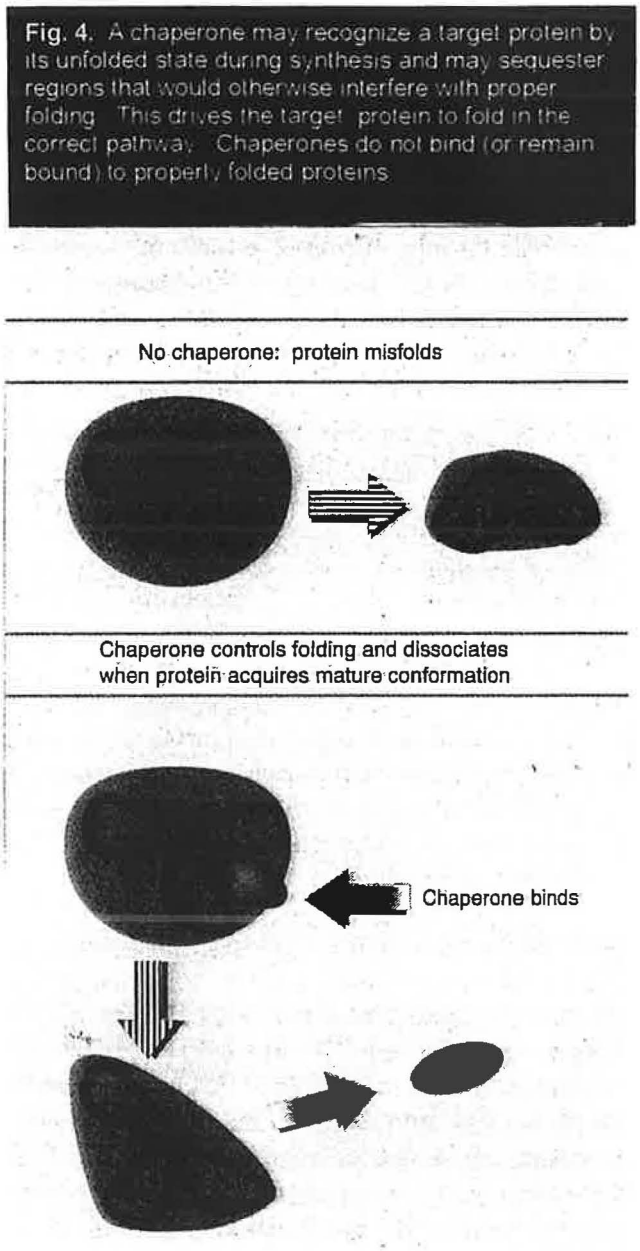


Fig. 4. A chaperone may recognize a target protein by its unfolded state during synthesis and may sequester regions that would otherwise interfere with proper folding. This drives the target protein to fold in the correct pathway. Chaperones do not bind (or remain bound) to properly folded proteins.

A reasonable hypothesis is that chaperone proteins are involved in maintaining the polyglutamine containing proteins or their truncated fragments from forming aggregates. It has been demonstrated that heat shock protein 70 (Hsp70) associates with polyglutamine containing proteins and their degradation fragments and promotes an ATP-dependent process of binding and release which extends the polypeptide segments. The truncated peptides are enriched in hydrophobic residues that are typically exposed in a non-native polyglutamine peptide, that is, fully or partially unfolded. The heat shock proteins by preventing aggregation of the polyglutamine residues prevent their forming aggregates that may progress from random coils to a parallel helical β-sheet (41,50). This structural conversion, if prevented, avoids the random conformation of oligomers that function as nuclei for rapid polymerization of nuclear amyloid-like fibrils (51). Heat shock protein 40 (Hsp40) has also been demonstrated to combine with the polyglutamine peptides. In support of this conclusion, there are several reports that increased expression of Hsp70 and Hsp40 chaperone systems can suppress the polyglutamine-induced neurotoxicity in Drosophila and in a mouse model of polyglutamine disease (52-54). Over expression of Hsp70 also suppresses polyglutamine-induced toxicity in spinal cerebellar ataxia-1 in cultured neurons and validates the role that molecular chaperones may play in preventing the development of neuronal degeneration (55).

The Role of Ubiquitin and the 20S and 19S Proteasome Complex (31,48,56-58)

Ubiquitin is recognized as a signal to cells for disposal of damaged protein and is an essential player in maintaining cell integrity. In this process, ubiquitin is activated first by the ubiquitin activating enzyme (E1) and then transferred to an ubiquitin conjugating enzyme (E2) which links activated ubiquitin to a protein in functional cooperation with an ubiquitin ligase which is a specificity factor (Fig. 5). Poly-ubiquitinated proteins are destined for digestion by a macromolecular machine, the 26S proteosome. This subcellular organelle consists of a barrel-shaped proteolytic core complex of 20S capped at both ends by 19S regulatory complexes. ATP-ases mediate unfolding and translocation of the substrate into the proteolytic chamber of the 20S complex and is accompanied by the release of ubiquitin molecules that are recycled. It has been demonstrated that polyglutamine inclusions stain positively on immunofluorescence for ubiquitin and for the 20S and 19S complexes. These findings provide strong support that the ubiquitin-proteosome system is involved in polyglutamine metabolism. When this protein disposal system is overwhelmed by the production of large numbers of polyglutamine fragments, they aggregate and form amyloid fibrils (41,42). The presence of intranuclear amyloid may lead to neuronal damage. Although the polyglutamine of huntingtin protein is cytoplasmic, it is transferred into the nucleus where intranuclear inclusions which characterize the disease are easily seen microscopically. Whether these nuclear aggresomes are the cause of neuronal cell death is not known.

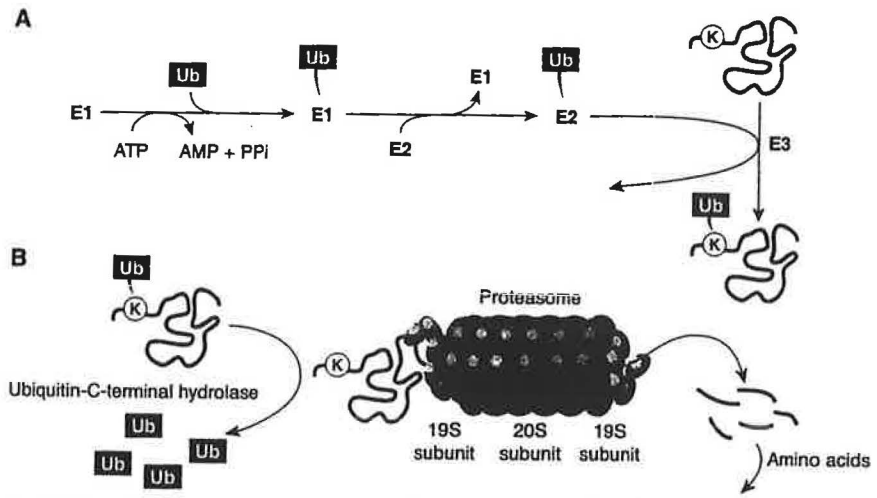


Fig. 5. The ubiquitin-proteasome system. **(A)** Proteins targeted for degradation are identified by covalent linkage to ubiquitin. Selective ubiquitination is accomplished by a series of enzymes (E1, E2, and E3) that constitute the ubiquitin ligase system. **(B)** Ubiquitinated substrates are recognized, unfolded, and degraded in an energy-dependent manner by the proteasome.

Possible Causes of Neuronal Cell Death

Every type of cell or tissue has a limited repertoire of response to noxious stimuli, a cascade of inter-related events progress to cell dysfunction and eventually cell death. It is expected that these responses are complex and that there are interactions between the components.

1) Protein misfolding

Polyglutamine-mediated neurologic diseases are one of the diseases caused by protein misfolding. The overproduction of polyglutamine peptides and their truncated fragments overwhelms the normal pathways for protein degradation and disposal. The formation of β sheets and peptide aggregates leads to amyloid formation which is translocated into the nucleus. Subsequent events summarized below contribute to irreversible neuronal

damage. It is clear that the polyglutamine peptides do not aggregate if Hsp70 and Hsp40 chaperonin levels are elevated in mutant cells. The prevention of amyloid formation is associated with survival of the neurons (5,54,59,60).

2) Caspase activation with apoptosis

The caspase cleavage of the polyglutamine region in exon 1 of the huntingtin protein activates caspases that may lead to activation of apoptosis. The caspase system is essential for carrying out the programmed cell death. Support for this hypothesis is provided by experiments in which caspase inhibitors prevent huntingtin protein cleavage. There is a marked reduction in toxicity of the polyglutamate protein. In transgenic mice, expressing human exon-1 of huntingtin with an expanded polyglutamine tract, the neuronal death is markedly reduced or delayed when animals are treated with a general caspase inhibitor Z-VAD-fmk or when crossed to mice expressing a dominant negative caspase 1 (44,61).

3) Polyglutamine diseases – a transcription disorder (62)

There is evidence that the formation of polyglutamine aggregates attract and interact with a variety of other proteins. There are at least 20 transcription related factors that have been demonstrated to interact with the polyglutamine peptide or their aggregates. The pathological interaction has been shown to repress gene expression. The nuclear localization of the polyglutamine amyloid interacting with transcription related factors has been proposed to play a role in early neuronal death.

Recent studies in animal models of HD show that mutant huntingtin deregulates transcriptional programs. Inhibiting the expression of the dopamine D_2 receptor may be particularly relevant to HD pathogenesis. Moreover, the multiprotein complexes that form in neuron nuclei with polyglutamine huntingtin contain the TATA box binding protein among several other transcription factors. The wide expression of HD protein and its derivations in the brain easily explains the compromised motor control and cognition.

4) The binding of oxidative enzymes such as NADPH oxidases and the attraction of inflammatory cells into the brain in HD and the neurodegenerative diseases such as Parkinson's and Alzheimer's disease provide adequate stimulation for the production of toxic oxygen radicals and free radicals that may modify the proteins and lipids of the neurons. Over a period of time, this injury might lead to neuronal death (63).

5) Polyglutamines may interfere with axonal transport in neurodegenerative diseases (64).

Neurons face a challenge in transporting newly synthesized proteins over long distances down their axons to the synapses, and return retrograde signals along the same path. In the case of motor neurons, the cell body may be as far as one meter away from the synapse. Alteration of either the anterograde or retrograde transport might be a reasonable explanation for dysfunction of a neuron and eventually its death. In amyotrophic lateral sclerosis, a degenerative disorder that particularly targets motor neurons, there is evidence of impaired axonal transport. Mutations in a protein, dynactin, required for transport, have been described in these patients. The polyglutamines reduce the level of dynactin by entrapment (64).

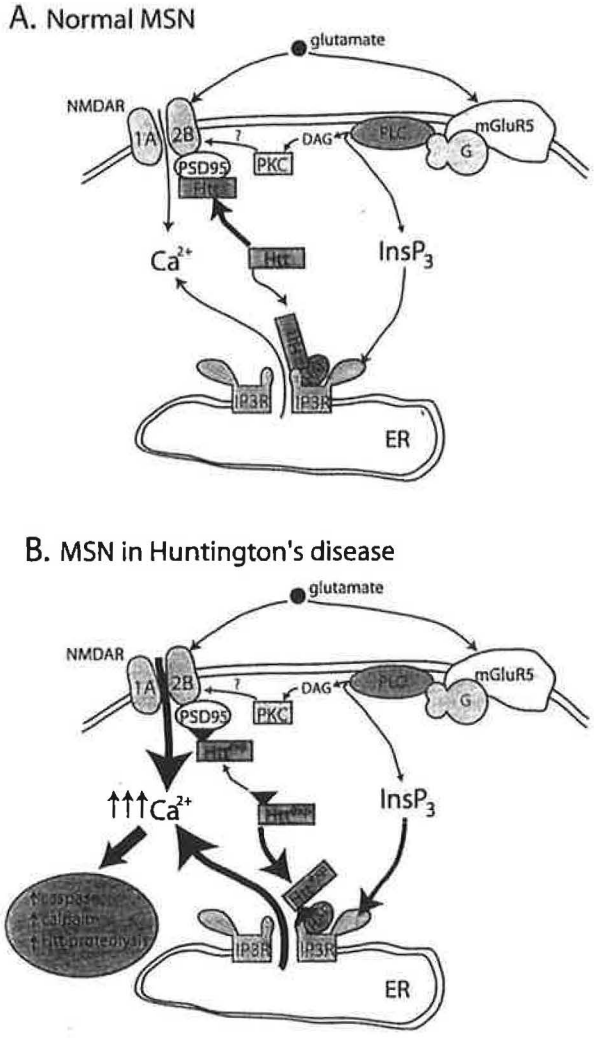
6) The excitotoxic hypothesis (65-68)

The excitotoxic hypothesis of neuronal loss in HD proposes that endogenously produced excitatory amino acids, for example, glutamine or closely related substances, physiologically involved in neurotransmission damage and kill neurons that are chronically exposed to their effects. The neurons affected are those that possess receptors for excitatory amino acids and include N-methyl-D-aspartate (NMDA). Glutamate is one of the prominent excitatory ligands for this receptor and stimulates increased neuronal metabolism. The receptor for the excitatory amino acids, including glutamate, are abundant in those areas of the brain that are affected in HD. Although this hypothesis has been championed by several investigators, it is difficult to see how the excitotoxic hypothesis can explain the chronicity of these diseases. However, recent elegant research in the Department of Physiology by Dr. Ilya Bezprozvanny has revived interest in this hypothesis.

7) Neuronal Calcium Signaling in HD (69)

Using *in vitro*, *in vivo* and cell culture of neurons, Dr. Bezprozvanny and his associates have demonstrated a dramatic effect of huntingtin protein and its polyglutamate mutant forms on calcium homeostasis in the medium spiny striatal neurons (MSNs) which are selectively targeted in HD. Figure 6A shows a normal medial striatal neuron (MSN) depicting two pathways for regulating calcium content. Both are stimulated by glutamate which is produced in corticostrial neurons. The glutamate receptor MGluR5 is a G protein – phospholipase C transduction complex which generates diacylglycerol (DAG) and inositol triphosphate ($InsP_3$), the second messengers in this cascade. Diacylglycerol (DAG) activates phosphokinase C which acts on the N-methyl D-aspartate receptor (NMDAR) to enhance its affinity for glutamate. Huntingtin protein interacts with a modulator of this receptor PSD95 to open the calcium channel. The inositol triphosphate ($InsP_3$) concurrently reacts with its receptor on the endoplasmic reticulum. Huntingtin protein with the huntingtin associated protein (HAP_1A) are important players in the functional complex during its interaction with the inositol

Fig. 6.



triphosphate receptor to release modest amounts of calcium from the endoplasmic reticulum (ER).

In Figure 6B, the effects of the polyglutamate expansion in huntingtins protein on calcium fluxes in the medium spiny neuron is depicted. Mutant huntingtin with the polyglutamine expansion (Htt^{exp}) enhances the signaling by two synergistic mechanisms. Mutant huntingtin (Htt^{exp}) enhances the calcium channel of NMDA receptor by decreasing the inhibitory effect of the modulator PSD95 and thereby increasing the influx of extracellular calcium. The mutant huntingtin (Htt^{exp}) binds huntingtin associated protein (HAP1A) tightly and the complex's affinity for inositol triphosphate receptor (IP_3R) is greatly enhanced. Therefore, the release of calcium from the endoplasmic reticulum (ER) is much greater. The net result is an elevation in intracellular calcium levels. The increased concentration of calcium triggers pathogenic pathways, for example, increased caspase and calpain activity, increased proteolysis of huntingtin, activation of apoptosis and neuronal degeneration. Abnormalities in ER-mediated calcium signaling are also described with mutations in presenilin 1 which has been linked to the development of premature Alzheimer's disease.

Elevation of the intracellular calcium level is recognized as a final common pathway for cell death. Ischemia, hypoxia and a large number of cellular injuries elevate intracellular calcium. Perturbed calcium homeostasis is an important step in promoting apoptosis in neurons (70). The demonstration by Dr. Bezprozvanny that the mutant huntingtin protein together with its huntingtin associated protein 1A can activate by two synergistic pathways elevated intracellular calcium not only suggests a new pathogenetic mechanism for HD but provides several targets for potential therapy.

It should be noted in the experiments conducted by Dr. Bezprozvanny that the normal huntingtin protein contained 23 glutamines while the mutant huntingtin contained 82 glutamines. This large polyglutamine expanse is in the range where HD is observed in young children. In acute experiments *in vitro* or in culture, rapid and dramatic effects are required. The more modest glutamine expansions observed in adult HD may explain the later onset of the disease. Programmed neuronal death (apoptosis) underline the pathogenesis of several neurodegenerative diseases including Alzheimer's, Parkinson's as well as Huntington's.

Therapy of Polyglutamine Associated Neurodegeneration

The therapy of HD and other neurodegenerative diseases depends on the development of substances that can ameliorate the abnormalities described above. For example, it has been shown that overexpression of molecular chaperones heat shock protein 70 and heat shock protein 40 can delay the development of Huntington's disease or reduce its severity in experimental animals such as mice. Inhibitors of the caspase proteolytic enzymes can also delay onset of HD in mice. However, this enzyme system is essential for normal cell turnover. It is clear that as our knowledge of the pathophysiology of diseases of misfolded proteins such as HD, spinal cerebellar ataxias, Alzheimer's disease, and Parkinson's disease increases, there may be rational ways of intervening therapeutically and, thereby, modifying the course of these diseases and perhaps in the future preventing them.

The role of huntingtin mutant protein with huntingtin associated protein type 1 (HAP1A) in increasing calcium levels in neurons offers several therapeutic targets. The inositol triphosphate receptor in ER and N-methyl D-aspartate receptor of subtype NR1A/NR1B is specifically sensitized by huntingtin polyglutamine mutants complexed with its partner HAP1A. Antagonists with specificity for these receptor subtypes might ameliorate HD. The m Glutamine R5 receptor is also a potential target for antagonists.

Conclusion

The expansion and translation of unstable triplet repeats in exons have provided a unique mutational mechanism for gain of function proteins. These polyglutamine expansions in specific proteins cause an abnormal conformation that may interact with a specific target. The particular protein with the polyglutamine streaks determines which neurons and areas of the brain are affected, for example, in HD and 11 types of spinocerebellar atrophy (SCA).

It is not known if the soluble polyglutamine proteins or the conversion to an insoluble amyloid structure initiate the pathogenesis of neuronal cell death. It would appear that a cascade of events may be involved in neuronal dysfunction. The events may differ in different cell types. However, identification of the participants in the initiating step by genetic and biochemical means remains crucial to achieving a complete understanding of polyglutamine-mediated pathogenesis in these disorders.

The participation of caspases in apoptotic pathways and the handling of proteins by the ubiquitin-proteosome-chaperone system are areas of intense interest. The role of polyglutamine expanded huntingtin protein with its associated companion HAP1A on calcium signaling is an exciting new discovery. Small molecules which modulate and inhibit receptors for inositol triphosphate, N-methyl D-aspartate of the NR1A/NR1B type and m glutamine R-5 are being investigated and are promising avenues for modifying disease.

Genetic analyses in humans and in several animal models have suggested criteria for recognizing and intervening in the initial pathogenetic step. Genetic analysis in model systems is a powerful approach that could reveal modifiers that suppress disease phenotypes. Evidence has been obtained in yeast, Drosophila and worms that molecular chaperones can inhibit polyglutamine aggregation and these studies should be valuable in detecting modifiers (71, 72-75, 76-79). Observations in humans also suggest the existence of genetic modifiers that affect the expression of polyglutamine-mediated neurodegenerative disorders (80-84).

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