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THE ROYAL SOCIETY

Polyglutamine pathogenesis

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An increasing number of neurodegenerative disorders have been found to be caused by expanding CAG triplet repeats that code for polyglutamine. Huntington's disease (HD) is the most common of these disorders and dentatorubral–pallidoluysian atrophy (DRPLA) is very similar to HD, but is caused by mutation in a different gene, making them good models to study. In this review, we will concentrate on the roles of protein aggregation, nuclear localization and proteolytic processing in disease pathogenesis. In cell model studies of HD, we have found that truncated N-terminal portions of huntingtin (the HD gene product) with expanded repeats form more aggregates than longer or full length huntingtin polypeptides. These shorter fragments are also more prone to aggregate in the nucleus and cause more cell toxicity. Further experiments with huntingtin constructs harbouring exogenous nuclear import and nuclear export signals have implicated the nucleus in direct cell toxicity. We have made mouse models of HD and DRPLA using an N-terminal truncation of huntingtin (N171) and full-length atrophin-1 (the DRPLA gene product), respectively. In both models, diffuse neuronal nuclear staining and nuclear inclusion bodies are observed in animals expressing the expanded glutamine repeat protein, further implicating the nucleus as a primary site of neuronal dysfunction. Neuritic pathology is also observed in the HD mice. In the DRPLA mouse model, we have found that truncated fragments of atrophin-1 containing the glutamine repeat accumulate in the nucleus, suggesting that proteolysis may be critical for disease progression. Taken together, these data lead towards a model whereby proteolytic processing, nuclear localization and protein aggregation all contribute to pathogenesis.

Keywords: Huntington's disease; dentatorubral–pallidoluysian atrophy; neuronal toxicity; transgenic mouse models

1. INTRODUCTION

The class of neurodegenerative diseases caused by expanding CAG repeats coding for polyglutamine includes Huntington's disease (HD), dentatorubral–pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA), and several forms of spino-cerebellar ataxia (SCA) (Ross 1995; Paulson & Fischbeck 1996; Gusella *et al.* 1997). Longer expanded repeats lead to earlier ages of onset (Duyao *et al.* 1993; Stine *et al.* 1993; Andrew *et al.* 1993, 1997; Nance 1997). HD is the most common of these disorders. It usually affects individuals in mid-adulthood but can begin anywhere from childhood to old age. Its symptoms include abnormal involuntary movements, such as chorea or dystonia, incoordination of voluntary movements, emotional symptoms and cognitive impairment leading to dementia. The disorder progresses gradually, leading to death about 15 to 20 years after onset of the disease. Patients who have adult onset DRPLA have a clinical picture very similar to that of HD (Ross *et al.* 1997). SBMA presents predominantly with muscle weakness. The spino-cerebellar ataxias can present a variety of syndromes, including ataxia, dystonia, incoordination, weakness, and other signs and symptoms.

In all of these disorders there is neuronal cell death in an overlapping set of brain regions, including the basal ganglia, cerebral cortex, brainstem nuclei and the cerebellum (Ross 1995). In HD, the corpus striatum (comprising the caudate and putamen) of the basal ganglia is most severely affected. In DRPLA, the deep cerebellar nuclei, including the dentate nucleus, show the greatest degeneration. Clinical features of the diseases are believed to result both from death of neurons and also from dysfunction of affected neurons before they die. In HD, the severity of symptoms generally correlates well with the extent of striatal neuronal cell death (Vonsattel *et al.* 1985; Myers *et al.* 1988). Death of neurons may be due to a form of cell death related to apoptosis, in which cells become condensed and die without substantial inflammation or injury to surrounding cells. Activation of intracellular proteases termed caspases is a usual feature of apoptotic cell death. All of the polyglutamine diseases appear to involve a genetic 'gain of function' mechanism at the protein level. The protein product of the HD gene is termed huntingtin, and the product of the DRPLA gene is termed atrophin-1.

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In all of the glutamine repeat diseases so far studied, inclusion bodies containing the protein product of the disease gene form in the nucleus of neurons (Ross 1997; Davies *et al*. 1997; Paulson *et al*. 1997; DiFiglia *et al*. 1997; Becher *et al*. 1998; Skinner *et al*. 1997). In addition, in some of the diseases there appears to be protein aggregation within neurons outside of the nucleus, for instance in structures called dystrophic neurites (DiFiglia *et al*. 1997). Thus, these diseases bear resemblance to other neurodegenerative diseases in which there are inclusion bodies or other proteinaceous deposits, including Alzheimer's disease with amyloid plaques and neurofibrillar tangles, and Parkinson's disease with Lewy bodies (Lansbury 1997).

In this review we will focus on three aspects of the pathogenesis of polyglutamine disorders: protein aggregation, nuclear localization and proteolytic processing. We will emphasize our own studies of HD and DRPLA, but also discuss the spino-cerebellar ataxias and spinal and bulbar muscular atrophy. For all of these disorders, cell and animal models are becoming increasingly important for elucidating pathogenic mechanisms.

2. AGGREGATION AND INCLUSION BODIES

(a) In vitro *studies*

The study of Scherzinger *et al*. (1997) was the first demonstration of the inherent ability of polyglutamine-containing protein to aggregate. The N-terminus of huntingtin was synthesized in bacteria and then released by proteolytic cleavage from the bacterial fusion protein to yield an N-terminal fragment (corresponding to exon-1) of about 90 amino acids. When this N-terminal fragment had a normal glutamine repeat it remained soluble. However, when the glutamine repeat length was in the range which causes HD, the protein formed aggregates which could be detected by collection on a filter membrane. Electron micrographs of these aggregates revealed a fibrillar or ribbon-like morphology. When the aggregates were stained with congo red dye they exhibited green birefringence characteristic of a β-pleated sheet confirmation. These characteristics are typical of amyloid fibrils of Alzheimer's disease or prion diseases.

Remarkably, formation of a β-pleated sheet had been previously predicted by Max Perutz, who hypothesized that glutamine repeats could form a so-called 'polar zipper' and result in non-covalent associations due to hydrogen bonds (Perutz 1994; Stott *et al*. 1995). This polar zipper was predicted to result when the glutamine repeat length exceeded a certain threshold. It was predicted to form either between two different molecules with glutamine repeats or within one molecule, forming a hairpin structure.

(b) *Neuropathological studies*

Protein aggregation in the form of intranuclear inclusions has been found in every polyglutamine disease examined to date. The first report was in Huntington's disease (DiFiglia *et al*. 1997). Huntingtin aggregation was found in intranuclear inclusions and dystrophic neurites in HD cortex and striatum. The density of the inclusions correlated with the length of the glutamine repeat at least in the cerebral cortex (Becher *et al*. 1998). Intranuclear inclusions have also been found in DRPLA (Becher *et al*. 1998; Igarashi *et al*. 1998), SCA3, SCA1 and other polyglutamine diseases (Paulson *et al*. 1997; Skinner *et al*. 1997; Holmberg *et al*. 1998; M. Li *et al*. 1998).

(c) *Animal and cell models*

Intranuclear inclusions were initially discovered in the first animal model of HD (Mangiarini *et al*. 1996; Davies *et al*. 1997). In this model, which was made with the short N-terminal fragment of huntingtin comprising exon-1 with a very long expanded repeat, intranuclear inclusions were seen densely in almost all regions of the brain. We have also seen intranuclear inclusions in a mouse model of HD with a slightly longer N-terminal truncation of huntingtin (the first 171 amino acids or N171) and a glutamine repeat length within the range commonly seen in juvenile HD patients (Schilling *et al*. 1999*a*). In this model, aggregates in neurites are also seen in several brain regions. Intranuclear inclusions have been seen in animal models of several other glutamine repeat disorders (Burright *et al*. 1995; Zoghbi 1996; Skinner *et al*. 1997; Reddy *et al*. 1998; Warrick *et al*. 1998; Faber *et al*. 1999; Jackson *et al*. 1998). Polyglutamine has also been inserted ectopically into an unrelated protein (HPRT) resulting in both a behavioural phenotype and intranuclear inclusions (Ordway *et al*. 1997).

In addition, polyglutamine aggregation has been seen in numerous cell models using a variety of different constructs (Ikeda *et al*. 1996; Paulson *et al*. 1997; Igarashi *et al*. 1998; Miyashita *et al*. 1998; Cooper *et al*. 1998; Li & Li 1998; Martindale *et al*. 1998; Merry *et al*. 1998; Lunkes & Mandel 1998; Hackam *et al*. 1998; Moulder *et al*. 1999). One recent study indicates that the extent of aggregation is correlated with the degree of toxicity, using an indirect measure of toxicity (Hackam *et al*. 1999).

However, two recent studies have raised the possibility that aggregation (or at least the large aggregates which can be seen as inclusion bodies) may not be the primary factor leading to cell toxicity. In one study, an animal model of SCA1 was generated using a construct similar to that used for the previous model (Burright *et al*. 1995; Klement *et al*. 1998). However, in this new model a deletion was made within a region of ataxin-1 which had been found to be a self-association domain (Burright *et al*. 1997; Klement *et al*. 1998). These mice developed ataxia and Purkinje cell pathology similar to the original SCA1 mice, at least in the early stages of the illness. However, no ataxin-1 aggregates were found (Klement *et al*. 1998). A second study involved a cell model using transient transfection of primary neurons in culture (Saudou *et al*. 1998). In this model intranuclear aggregates were formed. However, the presence of huntingtin aggregates did not correlate with cell toxicity. Under some circumstances there was an increase in aggregation but a decrease in toxicity. Thus, both of these experiments raise the possibility that polyglutamine protein aggregation, at least in large inclusions, may not be necessary for cellular toxicity.

3. NUCLEAR LOCALIZATION

Several studies suggest that the localization of the protein may be more important than whether it forms macroaggregates or not. In the study of Klement *et al*. (1998), an additional experiment was done. A construct

in which the nuclear localization signal was mutated was also used to generate the transgenic mice. These mice did not develop disease, suggesting that nuclear localization of the protein is critical for cell toxicity.

Similar results were obtained in the study of Saudou *et al*. (1998). Additional experiments in this study involved the use of an exogenous nuclear export signal on their huntingtin construct. Again, when the protein did not localize to the nucleus, there was dramatically less cellular toxicity.

These latter experiments may be complicated by the possibility that huntingtin has endogenous nuclear localization and nuclear export signals within its N-terminal portion. Our group has thus performed additional experiments with a shorter fragment of huntingtin (N63) which does not have any putative localization signals (Peters *et al*. 1999). Addition of an exogenous nuclear localization signal led to greater toxicity, while addition of an exogenous nuclear export signal led to less toxicity. By contrast, one cell model study (Hackam *et al*. 1999) suggests that nuclear localization does not increase toxicity, though toxicity was measured as a response to an exogenous stimulus, rather than directly. Animal model studies have not yet addressed in detail the issue of nuclear versus cytoplasmic localization of soluble polyglutamine proteins. Our own experience is that in animal models of both HD and DRPLA (see below), there is prominent diffuse label for the relevant protein (in addition to the intranuclear inclusions). Thus, while more work needs to be done, these results taken together suggest the possibility that nuclear localization is an important component of polyglutamine pathogenesis.

These studies raise the issue of the normal localization of not certain. Several polyglutamine-containing proteins are normally present at least, in part, in the nucleus, including the androgen receptor, as well as atrophin-1, ataxin-1 and ataxin-3 (Servadio *et al*. 1995; Tait *et al*. 1998; Trottier *et al*. 1998; Brooks *et al*. 1997; Brooks & Fischbeck 1995). The HPRT gene product used for the mouse model with ectopic polyglutamine expansion is small enough that it can diffuse into the nucleus without active transport (Ordway *et al*. 1997).

The normal cellular localization of huntingtin is not certain. Most studies have shown cytoplasmic localization (Sharp *et al*. 1995; Sapp *et al*. 1997; Ferrante *et al*. 1997; Aronin *et al*. 1995; DiFiglia *et al*. 1995). However, some studies have suggested a nuclear localization at least in some cells (Hoogeveen *et al*. 1993; De Rooij *et al*. 1996). In addition, the presence of putative nuclear export and nuclear localization signals raises the possibility that huntingtin may normally shuttle through the nucleus, perhaps in a regulated fashion.

What nuclear structures could the polyglutamine proteins be associated with? Early evidence suggests the possibility of association with structures in the nuclear matrix. Cell transfection studies indicate that ataxin-1 can associate with a protein present in the nuclear matrix called leucine-rich acidic nuclear protein (LANP) in a repeat length-dependent fashion. In addition, cotransfection of cells with ataxin-1 alters the distribution of the nuclear matrix associated PML protein (Skinner *et al*. 1997; Matilla *et al*. 1997). Ataxin-3 has also been reported to associate with the nuclear matrix (Tait *et al*. 1998). We have preliminary data suggesting that atrophin-1 associates with the nuclear matrix via an interaction with the ETO/MTG8 transcriptional co-repressor protein.

4. TRUNCATION OF POLYGLUTAMINE PROTEINS

Numerous cell model studies have indicated that truncated portions of polyglutamine proteins are more toxic than the full-length protein. In several different cell transfection paradigms, truncated N-terminal portions of huntingtin protein form more aggregates than longer or full-length huntingtin polypeptides in cells (Cooper *et al*. 1998; Martindale *et al*. 1998; Hackam *et al*. 1998; Saudou *et al*. 1998). Furthermore, small fragments of huntingtin are more likely to aggregate in the nucleus and appear to be more likely to cause cell toxicity (Cooper *et al*. 1998; Martindale *et al*. 1998; Hackam *et al*. 1998). Similarly for atrophin-1, ataxin-3 and the androgen receptor, short portions containing the polyglutamine tract are more toxic to cells than the full-length protein (Igarashi *et al*. 1998; Merry *et al*. 1998; Ikeda *et al*. 1996).

It is too early to be sure about the situation with mouse models, but the initial studies suggest that truncated fragments of huntingtin may be more toxic to mice. The very short N-terminal fragment used in the study of Mangiarini *et al*. (1996) produced a dramatic behavioural phenotype, although substantial neuronal cell death has not yet been documented in this model. High levels of expression of a full-length huntingtin construct led to relatively delayed expression of a behavioural phenotype and neuronal toxicity (Reddy *et al*. 1998). By contrast, expression of an N-terminal fragment (N171), even at very low levels of protein, caused a relatively early and dramatic behavioural phenotype and cell toxicity (Schilling *et al*. 1999*a*). However, because the promoters, mouse strains and repeat lengths are not exactly comparable, this issue is still not resolved.

The neuropathological data for huntingtin favour the idea that truncation of huntingtin protein is involved in the disease process. Post-mortem study of HD brains revealed the intranuclear inclusions only using antibodies directed at the N-terminus or epitopes close to the N-terminus. Epitopes several hundred amino acids away showed no reactivity (DiFiglia *et al*. 1997; Becher *et al*. 1998).

However, these studies do not reveal anything about the mechanism of truncation of the protein. *In vitro* studies have indicated that several of the polyglutamine proteins can be cleaved by the pro-apoptotic cysteine proteases termed caspases. The initial report for huntingtin suggested that this cleavage might be repeat length-dependent (Goldberg *et al*. 1996). However, subsequent experiments indicate that the full-length protein with an expanded repeat or a normal repeat are equally well-cleaved (Wellington *et al*. 1998). Caspase cleavage sites have been defined for huntingtin, atrophin-1 and the androgen receptor, though cleavage of huntingtin and atrophin-1 was much more efficient (Wellington *et al*. 1998). Huntingtin is cleaved most efficiently at amino acid 513 (Wellington *et al*. 1998). Atrophin-1 is cleaved by caspase 3 at position 109 (Miyashita *et al*. 1997; Wellington *et al*. 1998).

The relationship of these *in vitro* data with *in vivo* events is still uncertain. Identification of the mechanism of

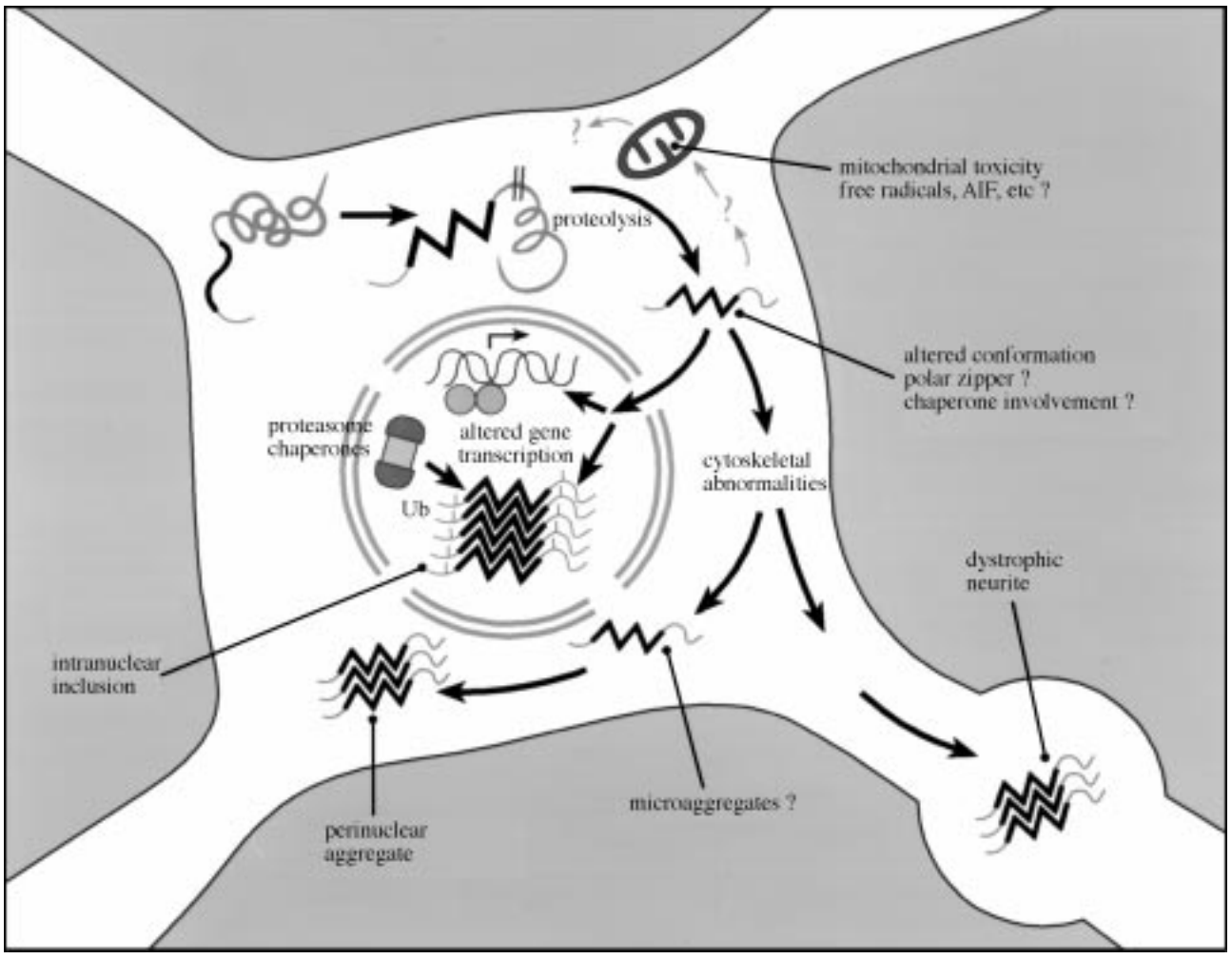

Figure 1. Current model for HD pathogenesis.

truncation *in vivo* has proved difficult. Cleavage of huntingtin was not described in the full-length cDNA transgenic mouse model of Reddy *et al*. (1998). Truncation has not been described in the SCA1 model, which also involves a full-length ataxin-1 protein (Burright *et al*. 1995).

Our recent studies with mice transgenic for a full-length atrophin-1 construct indicate that there is repeat length-dependent accumulation of truncated atrophin-1 products in the nuclei of neurons in this model (Schilling *et al*. 1999*b*). The mice express atrophin-1 with 65 glutamines under the control of the mouse prion promoter. These animals develop a dramatic progressive behavioural phenotype with incoordination, seizures, involuntary movements and weight loss progressing to early death. Post-mortem examination reveals both intranuclear inclusions and a diffuse nuclear label. Mice expressing atrophin-1 with a normal length repeat have no phenotype. Western blot analysis of nuclear fractions from these mice indicates that truncated atrophin-1 products accumulate selectively (or at least to much higher levels) in nuclei only in the mice expressing atrophin-1 with the expanded repeat. Full-length atrophin-1 is 1184 amino acids long and has an apparent molecular mass of about 180 kDa. In the cytoplasm, this is the predominant form of atrophin-1 present. By contrast, in the nucleus of the mutant animal, there is very little of the full-length protein, but there is a doublet of bands around 100 kDa which are reactive with an antibody to an epitope of atrophin-1 as well as with an antibody which detects expanded polyglutamine directly. These data suggest that truncated products of atrophin-1 accumulate in the nucleus in a repeat length-dependent fashion, though the mechanism is still uncertain.

5. OTHER ISSUES IN PATHOGENESIS

The preceding discussion has emphasized processes occurring within the nucleus. However, other effects may be relevant. The cytoplasmic inclusions observed in both mouse models and patients may contribute to neuronal dysfunction and perhaps the neuronal toxicity (DiFiglia *et al*. 1997; Gutekunst *et al*. 1999). In fact, in post-mortem brain tissue from most adult onset cases there appear to be fewer nuclear inclusions and more cytoplasmic inclusions and dystrophic neurites (DiFiglia *et al*. 1997). Long before the identification of the intranuclear inclusions, neurons from HD patients have been shown to have altered morphology using Golgi techniques (Graveland *et al*. 1985). These included not just the expected atrophic changes, but also changes suggestive of abnormal growth and plasticity of dendrites. How these fit in with current models has yet to be determined.

A number of studies have shown that huntingtin (and other glutamine repeat proteins) can interact with a variety of other proteins within the cell. These interactors include GAPDH (Burke *et al*. 1996; Koshy *et al*. 1996), HAP1 (Li *et al*. 1995; Li *et al*. 1998*a*), HIP1 (Kalchman *et al*. 1997; Wanker *et al*. 1997) and the E2-25k ubiquitin-conjugating enzyme (Kalchman *et al*. 1996). Some of these interactions can be modulated by the length of the glutamine repeat in huntingtin, though the extent to which this is relevant to pathogenesis is still uncertain. Most of these interactors appear to be predominantly cytoplasmic and relevance to HD pathogeneis is yet to be proven. Some of these interactors appear to be involved with vesicle transport, the cytoskeleton and other cellular transport activities (Colomer *et al*. 1997; Engelender *et al*. 1997; Li *et al*. 1998*b*; Wanker *et al*. 1997; Kalchman *et al*. 1997). Both huntingtin and HAP1 can undergo axonal transport in neurons (Block-Galarza *et al*. 1997). Several huntingtin interactors have recently been identified which are nuclear proteins, but the relevance of these to pathogenesis has not yet been determined (Faber *et al*. 1998; Jones *et al*., this issue).

It has been proposed that huntingtin toxicity may relate to cellular phosphorylation pathways (Liu 1998; Liu *et al*. 1997), but this remains to be demonstrated in animal models or human pathological material. Recent data suggest that huntingtin can activate initiator caspases (perhaps via dimerization) triggering cell death pathways (Sanchez & Yuan 1998). Transfection of cells with polyglutamine fusion proteins can activate caspases (Moulder *et al*. 1999).

6. CONCLUSIONS

The field of polyglutamine pathogenesis is making rapid progress. A number of mechanisms have been identified, but how they all fit together is still uncertain. The *in vitro* data and the repeat length dependence of aggregation are highly suggestive that aggregation may be involved in some way. Microaggregates may be more relevant to toxicity than macroaggregates or inclusion bodies. Proteolytic processing may be involved, though the mechanisms are unknown.

Most interestingly, nuclear mechanisms are now being implicated. Whether these involve interaction with transcription factors or other molecules such as splicing factors is unknown. It is provocative that both huntingtin and atrophin-1 can interact with proteins in nuclear co-repressor complexes (NCoR and ETO, respectively). The androgen receptor, which undergoes polyglutamine expansion in SBMA, is itself a steroid hormone receptor which can functionally interact with both nuclear co-activator and co-repressor complexes. The mice made in the Bates's laboratory have dramatic alterations in levels of a number of receptor proteins, suggestive of alterations in gene transcription, though the mechanism is still unknown (Cha *et al*. 1998). The relationship between ataxin-1 and PML-oncogenic domains (PODs), which may be transcriptional regulatory domains, is provocative and needs to be followed up.

A model of some of the mechanisms discussed in the text is shown in figure 1, focusing on the possible neuronal effects of huntingtin. No doubt the model will need to be modified as further data are generated.

A better understanding of pathogenic mechanisms will be critical for the development of rational therapeutics. The *in vitro*, cellular and animal models described above will be critical for screening and then for further testing possible therapeutic compounds which can then be tried in HD patients.

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