PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Evidence for both nucleus and cytoplasm as subcellular sites of pathogenesis in Huntington'sdisease in cell culture and in transgenic mice expressing mutant huntingtin

Abigail S. Hackam, J. Graeme Hodgson, Roshni Singaraja, Taiqi Zhang, Lu Gan, Claire-Anne Gutekunst, Steven M. Hersch and Michael R. Hayden

Phil. Trans. R. Soc. Lond. B 1999 **354**, 1047-1055
doi: 10.1098/rstb.1999.0457

THE ROYAL SOCIETY

Evidence for both the nucleus and cytoplasm as subcellular sites of pathogenesis in Huntington's disease in cell culture and in transgenic mice expressing mutant huntingtin

Abigail S. Hackam[1,2], J. Graeme Hodgson[1,2], Roshni Singaraja[1,2], Taiqi Zhang[1,2], Lu Gan[1,2], Claire-Anne Gutekunst[3], Steven M. Hersch[3] and Michael R. Hayden[1,2*]

[1]*Centre for Molecular Medicine and Therapeutics, 980 West 28th Avenue, Vancouver, British Columbia, Canada V5Z 4H4*
[2]*Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada V6T IZ4*
[3]*Emory University School of Medicine, Atlanta, GA 30322, USA*

A unifying feature of the CAG expansion diseases is the formation of intracellular aggregates composed of the mutant polyglutamine-expanded protein. Despite the presence of aggregates in affected patients, the precise relationship between aggregates and disease pathogenesis is unresolved. Results from *in vivo* and *in vitro* studies of mutant huntingtin have led to the hypothesis that nuclear localization of aggregates is critical for the pathology of Huntington's disease (HD). We tested this hypothesis using a 293T cell culture model system by comparing the frequency and toxicity of cytoplasmic and nuclear huntingtin aggregates. Insertion of nuclear import or export sequences into huntingtin fragments containing 548 or 151 amino acids was used to reverse the normal localization of these proteins. Changing the subcellular localization of the fragments did not influence their total aggregate frequency. There were also no significant differences in toxicity associated with the presence of nuclear compared with cytoplasmic aggregates. These studies, together with findings in transgenic mice, suggest two phases for the pathogenesis of HD, with the initial toxicity in the cytoplasm followed by proteolytic processing of huntingtin, nuclear translocation with increased nuclear concentration of N-terminal fragments, seeding of aggregates and resultant apoptotic death. These findings support the nucleus and cytosol as subcellular sites for pathogenesis in HD.

Keywords: Huntington's disease; aggregates; pathogenesis; *in vitro*; animal model

1. INTRODUCTION

The expansion of a polymorphic CAG tract encoding glutamine is the causative mutation in eight human neurodegenerative diseases, including Huntington's disease (HD), dentatorubral–pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA), and spinocerebellar ataxia (SCA) types 1, 2, 3, 6 and 7 (Ross 1997; Andrew *et al.* 1997). Each disease affects specific populations of neurons and results in a characteristic clinical phenotype. Additionally, the mutant genes responsible for these diseases have no sequence similarity except for the CAG tracts. However, there may be a common step in the pathogenic pathways of the diseases that involve novel properties of the expanded CAG tract.

Several reports in the past year have described intracellular protein inclusions, or aggregates, within and outside the nuclei of cells expressing proteins with expanded polyglutamine tracts (reviewed in Ross 1997) (Lunkes & Mandel 1997; Hackam *et al.* 1998*b*). Several lines of evidence from patient samples, transgenic mice and cell culture models suggest that the aggregates are associated with the pathology of CAG expansion diseases. First, the inclusions are only observed in the brains of individuals carrying the disease allele, and are present predominantly in regions and neuronal populations affected by the disease (DiFiglia *et al.* 1997; Gourfinkel-An *et al.* 1998; Sapp *et al.* 1997; Paulson *et al.* 1997; Igarashi *et al.* 1998; Skinner *et al.* 1997; Becher *et al.* 1998; Li *et al.* 1998). Second, neuronal inclusions have also been observed in mice transgenic for genes with expanded CAG tracts, which develop inclusions prior to the onset of neurological symptoms (Davies *et al.* 1997; Ordway *et al.* 1997; Skinner *et al.* 1997). Third, increasing frequency of aggregates is associated with increasing toxicity in *in vitro* models of HD, DRPLA and SBMA (Ellerby *et al.* 1999; Hackam *et al.* 1998*a*; Igarashi *et al.* 1998). Further, decreasing the frequency of aggregates *in vitro* results in reduced toxicity (Ellerby *et al.* 1999; Igarashi *et al.* 1998). However, despite these data suggesting a causal relationship, none can differentiate between aggregates as being crucial to pathogenesis, from being markers of pathology.

Recent attention has focused on the nucleus as the primary site of pathogenic changes in polyglutamine

*Author for correspondence (mrh@cmmt.ubc.ca).

expansion diseases. One line of evidence for nuclear involvement is the exclusive nuclear localization of aggregates in post-mortem brains in SCA-1 (Skinner *et al.* 1997), SCA-3 (Paulson *et al.* 1997), SCA-7 (Holmberg *et al.* 1998) and DRPLA (Igarashi *et al.* 1998; Becher *et al.* 1998). Intranuclear aggregates were also observed in mice transgenic for a hypoxanthine phosphoribosyl transferase (HPRT) gene containing an expanded polyglutamine stretch and in mice expressing exon 1 of the *HD* gene (Davies *et al.* 1997; Ordway *et al.* 1997). Unusual nuclear morphology, including irregular indentation of the nuclear membrane, increased pore density and chromatin condensation have also been described in transgenic mice (Davies *et al.* 1997; Skinner *et al.* 1997) and patient brains (Tellez-Nagel *et al.* 1974; Bots & Bruyn 1981).

Aggregates were found to be exclusively nuclear in one study of adult and juvenile HD patients (Becher *et al.* 1998). However, other studies using different antibodies have identified both intranuclear and cytosolic accumulations of huntingtin in brain tissue of HD patients (DiFiglia *et al.* 1997; Gourfinkel-An *et al.* 1998). In brains of severely affected juvenile HD patients, huntingtin-containing inclusions were identified within the nuclei of neurons in the cortex and striatum (DiFiglia *et al.* 1997). Adult HD patients also displayed extranuclear accumulations of huntingtin in dystrophic neurites and perikarya (DiFiglia *et al.* 1997; Gourfinkel-An *et al.* 1998). Neurons containing cytoplasmic accumulation were more frequent in adult patients than neurons with nuclear inclusions (DiFiglia *et al.* 1997), indicating that cytoplasmic aggregates can also be cytotoxic. Therefore, whether the specific subcellular localization of the huntingtin aggregates is a contributing factor to the pathology in HD is not clear.

In vitro studies have demonstrated that nuclear localization of huntingtin aggregates is influenced by the length of the protein (Hackam *et al.* 1998*a*; Cooper *et al.* 1998). Small huntingtin proteins are both nuclear and cytoplasmic whereas larger proteins are only cytoplasmic, suggesting that passive diffusion plays a role in intracellular localization. Proteolytic cleavage would therefore be necessary to reduce the large full-length huntingtin protein to a fragment that is capable of diffusion through the nuclear pores (< 60 kDa) (Görlich & Mattaj 1996). However, we also identified a basic amino-acid-rich sequence within the N-terminus of huntingtin with significant homology to a functional nuclear localization sequence (NLS) (Hackam *et al.* 1998*a*), which suggested that huntingtin may also enter the nucleus by active transport. In order to determine the mode of nuclear transport, we assessed the ability of the predicted NLS to transport mutant huntingtin into the nucleus.

The predominance of nuclear aggregates has led us and others to postulate the nucleus as the site of pathology in HD (Ross 1997; Hackam *et al.* 1998*b*; Lunkes & Mandel 1997; Davies *et al.* 1997; Paulson *et al.* 1997; DiFiglia *et al.* 1997). This would suggest that a key event in the pathogenesis of HD is the translocation of truncated mutant huntingtin to the nucleus, where it exerts its toxic effect. An unanswered question is whether these aggregates may be similarly toxic outside the nucleus. Additionally, toxicity could be mediated by cell-specific vulnerability to mutant huntingtin and aggregate formation in the nucleus, or by interaction with specific nuclear proteins.

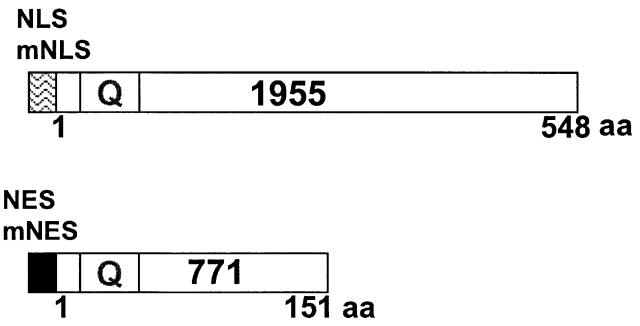

Figure 1. cDNA constructs used in this study. The 771 bp (1–151 amino acids) and 1955 bp (1–548 amino acids) constructs are represented. The position of the polyglutamine tract, either 15 or 128 units, is indicated by the letter Q. The position of the first huntingtin residue is indicated by the number 1. An NLS or mutant NLS (dashed box) was ligated into the 5′-end of the 1955-15 and 1955-128 constructs. An NES or mutant NES (filled box) was ligated into the 5′-end of the 771-15 and 771-128 constructs.

In this study we have designed experiments to specifically investigate the subcellular sites of toxicity in HD. Our results indicate both the nucleus and cytoplasm represent sites of toxicity of huntingtin.

2. RESULTS

(a) *Altering the location of aggregates formed by the 1955-128 protein does not influence toxicity*

To assess whether the subcellular site of aggregation influences toxicity, we altered the localization of aggregates formed by the 1955-128 protein and compared their toxicity in different subcellular locations. The 1955-128 huntingtin fragment includes amino acids 1 to 548, corresponding in size to the fragment produced by caspase 3 cleavage (Goldberg *et al.* 1996; Wellington *et al.* 1998), and contains 128 polyglutamines. This fragment has been consistently identified in transfected cells undergoing stress (Martindale *et al.* 1998), suggesting that it may be a stable fragment produced from full-length huntingtin.

The cytoplasmic perinuclear location of 1955-128 aggregates was changed to a nuclear location by the addition of an NLS. The NLS from the SV40 large T antigen (PKKKRKV) was inserted into the N-terminus of 1955-128, forming 1955-128-NLS (figure 1). Immunofluorescence of cells transfected with the 1955-128 constructs was used to quantify their subcellular localization (table 1). In several cell types and using multiple anti-huntingtin antibodies, the 1955-128 protein is exclusively cytoplasmic (Martindale *et al.* 1998) (figure 2). The 1955-128 protein has a predicted molecular mass of 73 kDa, which is too large to enter the nucleus by passive diffusion.

In contrast to 1955-128, 100% of cells expressing 1955-128-NLS protein had nuclear huntingtin stain (figure 2), indicating that the ectopic NLS is functional when introduced into huntingtin. The total frequency of aggregates was similar between the 1955-128 and 1955-128-NLS proteins (table 1). However, the proportion of aggregates in the nucleus differed between the proteins, with 0% nuclear aggregates for 1955-128 and 100% for 1955-128-NLS, in parallel with the total nuclear stain.

Table 1. *Frequency and subcellular localization of huntingtin aggregates*

construct	nuclear aggregates (%)	cytoplasmic aggregates (%)	total number of cells with aggregates (%)[a]
1955-128	0 ± 0	100 ± 0	7.8 ± 2.6
1955-128-NLS	100 ± 0	0 ± 0	5.4 ± 1.7
1955-128-mNLS	0 ± 0	100 ± 0	2.7 ± 1.5
771-128	49.2 ± 15.7	50.8 ± 15.7	75.4 ± 10.7
771-128-NES	2.0 ± 2.0	98.0 ± 2.0	51.3 ± 16.5
771-128-mNES	51.6 ± 18.0	48.4 ± 18.0	68.4 ± 19.0

[a]There were no significant differences between the total aggregate frequency between the different 1955 proteins or 771 proteins, except between 1955-128 and the control protein 1955-128-mNLS ($p < 0.01$).

A mutant NLS (PAAAAAV) was also inserted into 1955-128 (forming 1955-128-mNLS), to control for any effect of an introduced peptide on toxicity and aggregate formation. Immunofluorescence on transfected cells showed that the 1955-128-mNLS protein had 0% nuclear stain (figure 3). Despite the differences in subcellular localization of 1955-128-mNLS and 1955-128-NLS proteins, there was no significant difference in the frequency of aggregates (table 1).

In the 293T cell model, expression of proteins with an expanded polyglutamine tract results in an increase in susceptibility to apoptotic stress from treatment with a sub-lethal concentration of tamoxifen (Ellerby *et al.* 1999*b*; Hackam *et al.* 1998*a*; Martindale *et al.* 1998; Ellerby *et al.* submitted). The resultant cell death is quantified by a monotetrazolium (MTT) assay, a standard apoptosis assay (Carmichael *et al.* 1987) that is a sensitive indicator of cell viability. Mock-transfection cells and LacZ-transfected cells, both treated with tamoxifen, are used as controls. To compare the toxicity of aggregates from the same sized huntingtin proteins in different cellular compartments, 293T cells were transfected with the 1955-128 constructs containing the functional and mutant NLS sequences. As shown in table 2, there was no significant difference in cell death due to expression of the 1955-128 and 1955-128-NLS proteins ($n = 5$), indicating that the toxicity of nuclear aggregates was the same as that of cytoplasmic aggregates. The control, 1955-128-mNLS, which forms cytoplasmic aggregates, also had the same toxicity as 1955-128 (table 2), indicating that addition of a peptide had no influence on toxicity. Western blotting demonstrated equivalent expression levels for each construct (data not shown). Therefore, aggregates generated by 1955-128 proteins have similar toxicity regardless of their localization.

(b) *Moving 771-128 aggregates out of the nucleus does not change their toxicity*

The 771-128 protein has the highest frequency of aggregates in the nucleus, compared with other huntingtin fragments assessed (Hackam *et al.* 1998*a*). The 771-128 protein is also extremely toxic to cells in the presence of an apoptotic stress. To assess further the influence of subcellular localization of huntingtin on toxicity, we created a construct that brought the 771-128 protein out of the nucleus, and compared the toxicity of nuclear and cytoplasmic aggregates.

A nine-residue nuclear export sequence (NES: LALKLAGLDI) from the cAMP-dependent protein kinase inhibitor was inserted into 771-128, forming 771-128-NES (figure 1). Immunofluorescence studies on 293T cells expressing the 771-128 proteins are shown in figure 3. At 36 h post-transfection the per cent of cells expressing 771-128 with nuclear aggregates was 49%. In contrast, the per cent of nuclear aggregates of the 771-128-NES protein was < 2% (table 1), indicating that the NES is functional when inserted into huntingtin. The total frequency of aggregates formed by 771-128 and 771-128-NES was not significantly different (table 1).

At 48 h post-transfection, the proportion of nuclear aggregates was higher for the 771-128-NES protein than at 36 h, most likely because passive diffusion into the nucleus occurred at a greater rate than the energy-dependent active transport out of the nucleus using the NES. A time-course assessment of aggregate formation determined that the highest proportion of cytoplasmic aggregates formed by 771-128-NES occurred at 36 h post-transfection (data not shown). Therefore, toxicity studies for this set of experiments were performed at 36 h post-transfection. Since the analysis of 771-128 was performed at 36 h, while the analysis of the 1955-128 proteins was at 48 h, the aggregate frequency and cell viabilities between these experiments cannot be compared.

To control for addition of a peptide, a 771-128-mNES construct was created with a mutant NES, AAAKAAGADA. Immunofluorescence studies demonstrated that the proportion of aggregates in the nucleus was 52% for 771-128-mNES, which is similar to that of the 771-128 protein, but differs substantially from the 771-128-NES protein. The total frequency of aggregates formed by the 771-128-mNES protein was not statistically different from either the 771-128 or 771-128-NES protein (table 1).

The toxicity of aggregates formed by the 771-128 proteins in different subcellular locations was compared at 36 h (table 3). There was no significant difference in toxicity between aggregates formed by 771-128 and 771-128-NES proteins ($n = 5$). In addition, there was no difference in toxicity between aggregates formed by 771-128 and 771-128-mNES, indicating that addition of a peptide had no effect ($n = 5$). There was also no difference in toxicity between the 771 proteins at 48 h, although overall toxicity at 48 h was greater than at 36 h due to higher protein expression (data not shown; Hackam *et al.* 1998*a*). Western blotting confirmed equivalent expression levels (data not shown). These results show that the 771-128 aggregates in the nucleus were associated with levels of toxicity similar to those of aggregates outside the nucleus.

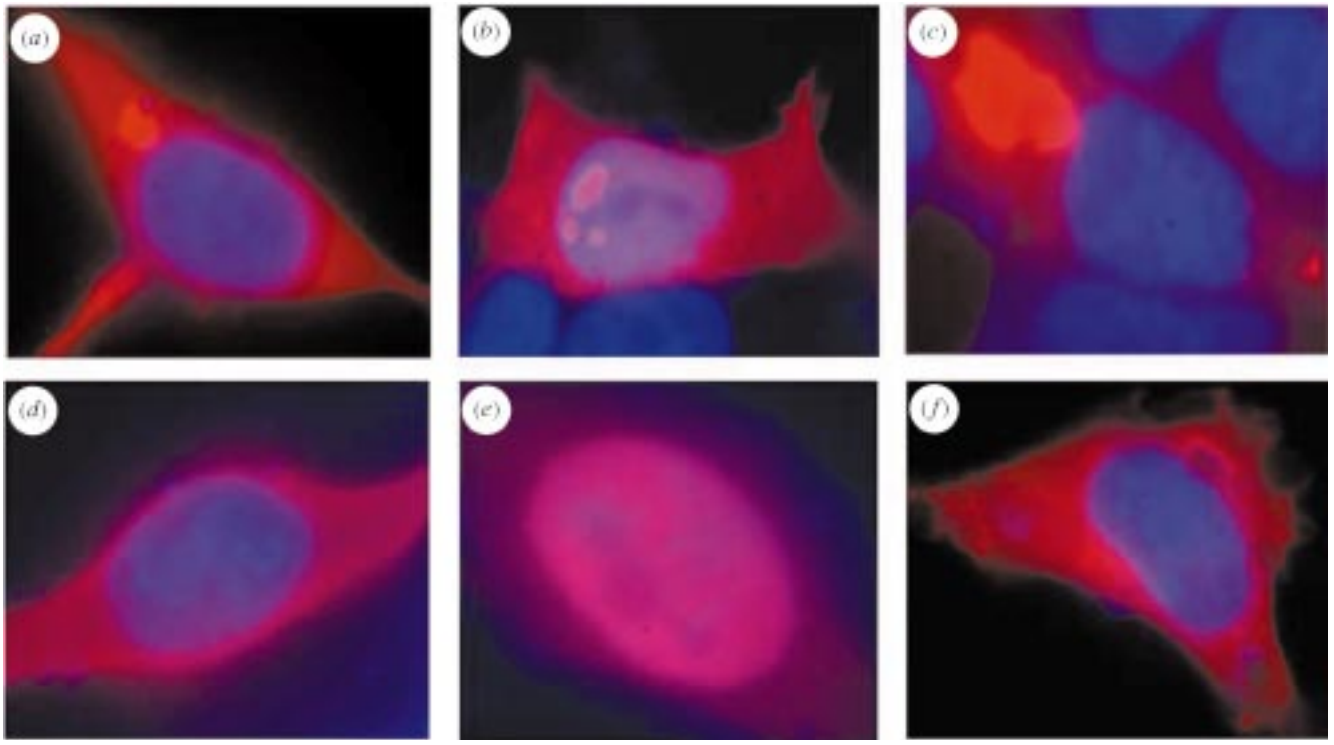

Figure 2. The localization of huntingtin is altered by the addition of an active NLS sequence. Huntingtin, detected by MAb 2166, appears as red stain, the nucleus is counter-stained in blue. Nuclear huntingtin stain is pink when the red stain is overlapped with blue. The huntingtin aggregates appear as large clumped masses, easily differentiated from normal diffuse stain. The size of the aggregates varied from cell to cell, but there was no consistent size difference between aggregates formed by the protein products of the three 1955-128 constructs. The aggregates formed by the 1955-128 protein are cytoplasmic (*a*), by the 1955-128-NLS protein, with an active NLS, are nuclear (*b*), and by the 1955-128-mNLS control protein, containing a mutant NLS, are cytoplasmic (*c*). The protein product of the 1955-15 construct is cytoplasmic (*d*), the 1955-15-NLS protein, with an active NLS, is nuclear (*e*), and the 1955-15-mNLS control protein, containing a mutant NLS, is cytoplasmic (*f*).

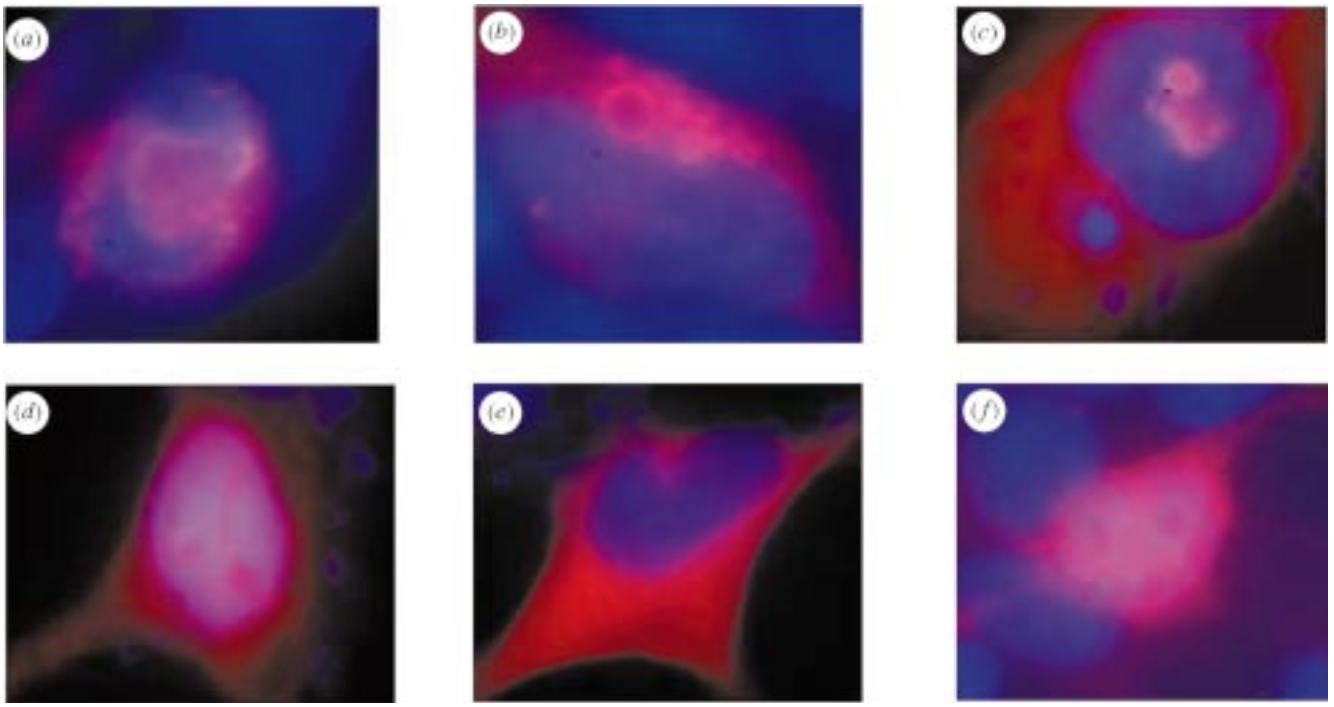

Figure 3. The localization of huntingtin is altered by the addition of an active NES sequence. Huntingtin is shown in red, the nucleus is counter-stained in blue, and nuclear huntingtin stain is pink when the red stain is overlapped with blue. The size of the aggregates varied from cell to cell, but there was no consistent size difference between aggregates formed by the three 771-128 proteins. 771-128 protein forms nuclear aggregates (*a*), 771-128-NES protein, containing an active NES, forms predominantly cytoplasmic aggregates (*b*), and the 771-128-mNES control protein, containing a mutant NES, forms nuclear aggregates (*c*). In contrast, 771-15 protein is nuclear (*d*), 771-15-NES protein, containing an active NES, is cytoplasmic (*e*), and the 771-15-mNES control protein, containing a mutant NES, is nuclear (*f*).

Table 2. *Importing huntingtin into the nucleus does not change its toxicity*

(Apoptosis assessed at 48 h post-transfection. The 1955-128 proteins are significantly more toxic than the 1955-15 proteins at $p < 0.001$ ($n = 5$).)

protein	toxicity relative to LacZ (%)
1955-128	71.88 ± 1.36
1955-128-NLS	72.66 ± 1.37
1955-128-mNLS	72.70 ± 0.67
1955-15	92.60 ± 0.91
1955-15-NLS	92.30 ± 0.53
1955-15-mNLS	92.00 ± 1.05

Table 3. *Exporting huntingtin from the nucleus does not change its toxicity*

(Apoptosis was assessed for the 771 constructs at 36 h post-transfection instead of 48 h to maximize the proportion of 771-128-NES in the cytoplasm. The 771-128 proteins are significantly more toxic than the 771-15 proteins at $p < 0.001$ ($n = 5$).)

protein	toxicity relative to LacZ (%)
771-128	72.13 ± 0.95
771-128-NES	72.06 ± 1.03
771-128-mNES	71.81 ± 0.53
771-15	90.24 ± 0.85
771-15-NES	90.67 ± 0.99
771-15-mNES	90.41 ± 0.68

Therefore, the subcellular localization of 771-128 aggregates does not influence susceptibility to cell death.

(c) *The subcellular localization of huntingtin fragments with wild-type polyglutamine tracts does not influence toxicity*

In the 293T cell model, huntingtin with longer CAG tracts (128 glutamines) has significantly higher toxicity than that with shorter tracts (15 glutamines) (Hackam *et al.* 1998*a*; Martindale *et al.* 1998). This repeat-length dependence on toxicity occurs for both full-length huntingtin and truncated huntingtin fragments (Hackam *et al.* 1998*a*). However, it has been noted previously that cells expressing truncated huntingtin containing 15 polyglutamines have an increased susceptibility to cell death (albeit less than seen with mutant huntingtin), which may represent a role for wild-type huntingtin in the regulation of cell viability (Hackam *et al.* 1998*a*; Martindale *et al.* 1998). Similar findings of toxic effects for other wild-type truncated polyglutamine-containing proteins have been observed (Ellerby *et al.* 1999*a*).

In 293T cells, the small 771-15 protein has been shown to be more toxic than the 1955-15 protein (Hackam *et al.* 1998*a*). The 771-15 is predominantly nuclear in transfected cells, whereas 1955-15 is exclusively cytoplasmic. Unlike their counterparts with 128 glutamines, the wild-type proteins do not form aggregates. Therefore, the greater toxicity of 771-15 over 1955-15 is not due to aggregate formation but could be associated with its nuclear localization. To test the influence of subcellular location on the toxicity of the wild-type huntingtin fragments, we altered the normal localization of 771-15 and 1955-15.

Table 4. *The proportion of cells with nuclear or cytoplasmic huntingtin is presented as a per cent of the total number of cells expressing huntingtin*

construct	nuclear localization (%)	cytoplasmic localization (%)
1955-15	0 ± 0	100 ± 0
1955-15-NLS	100 ± 0	0 ± 0
1955-15-mNLS	0 ± 0	100 ± 0
771-15	76.0 ± 8.6	24.0 ± 8.6
771-15-NES	58.0 ± 12.5	42.0 ± 12.5
771-15-mNES	75.0 ± 9.2	25.3 ± 9.2

To alter the localization of the 1955-15 protein, the SV40 NLS sequence was inserted into 1955-15, forming 1955-15-NLS (figure 1). Immunofluorescence of the 1955-15 and 1955-15-NLS is shown in figure 2. At 48 h post-transfection, the per cent of cells with nuclear stain was 0% for 1955-15 and 100% for 1955-15-NLS (table 4). The control peptide, encoding a non-functional NLS, was also inserted into 1955-15, forming 1955-15-mNLS. The 1955-15-mNLS protein had 0% nuclear stain (figure 2, table 4). Although the subcellular localizations were obviously different, when tested for toxicity there was no significant difference between the 1955-15, 1955-15-NLS and 1955-15-mNLS proteins (table 2). Therefore, for the same sized proteins, the wild-type huntingtin fragments in the cytoplasm have the same susceptibility to cell death as nuclear huntingtin.

However, the toxicity associated with the 1955-128 proteins was significantly greater than that seen with the 1955-15 proteins ($p < 0.001$, $n = 5$ ANOVA and Newman–Keuls test) (table 2). This result demonstrates the potent influence of increasing CAG repeat length on susceptibility to cell death, as shown previously (Hackam *et al.* 1998*a*; Martindale *et al.* 1998).

To change the predominantly nuclear 771-15 to a predominantly cytoplasmic protein, the NES sequence was inserted into 771-15, forming 771-15-NES (figure 1). At 36 h post-transfection the per cent of cells with 771-15 in the nucleus was 76%. The number of cells with nuclear protein was reduced to 58% for 771-15-NES. The control peptide encoding a non-functional NES was also inserted into 771-15, to create 771-15-mNES. The per cent of nuclear stain for 771-15-mNES was 75%, similar to that with the parental protein 771-15 (figure 3, table 4). Consistent with the experiments described above, altering the localization of the 771-15 proteins also did not result in significant differences in toxicity (table 3). The 771-15 protein, which is predominantly nuclear, had similar toxicity as the 771-15-NES protein, which is predominantly in the cytosol. Furthermore, the 771-15-NES protein had similar toxicity to that of the product of the control construct, 771-15-mNES, with predominantly nuclear localization. Therefore, the subcellular localization of the 771-15 proteins does not influence their toxicity. In addition, there was a significant decrease in cell death associated with expression of the 771-15 proteins compared with the 771-128 proteins ($p < 0.001$, $n = 5$), as

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

described for the 1955 proteins. Since the assessment of viabilities of the products of the 771-15 constructs was performed at 36 h, and the 1955-15 analysis was at 48 h, the values cannot be compared between experiments.

3. DISCUSSION

(a) *The subcellular localization of huntingtin aggregates does not influence toxicity*

Several lines of evidence have suggested that HD is a disease of the nucleus. Intranuclear inclusions are the predominant marker in affected patients (DiFiglia *et al.* 1997; Becher *et al.* 1998; Gourfinkel-An *et al.* 1998), and mice transgenic for huntingtin exon 1 develop nuclear aggregates prior to the onset of debilitating neurological symptoms (Davies *et al.* 1997). Furthermore, increasing toxicity of successively smaller huntingtin fragments in our cell culture model was associated with the formation of nuclear aggregates (Hackam *et al.* 1998*a*). Increasing toxicity *in vitro* was also associated with increased aggregate frequency (Hackam *et al.* 1998*a*). In this study, we have directly addressed the questions of whether the site of huntingtin influences its toxicity, and how huntingtin enters the nucleus.

We altered the subcellular localizations of huntingtin protein fragments with the addition of NLS and NES peptides, while the frequency of aggregates remained equivalent. The cytoplasmic protein 1955-128 was changed to a protein that forms nuclear aggregates. The 771-128 protein, which forms predominantly nuclear aggregates, was altered to form predominantly cytoplasmic aggregates. Our results in this cell culture model indicate that toxicity is not dependent on the subcellular localization of aggregates, but toxicity is associated with the frequency of aggregate formation.

(b) *The cell culture model can mimic* in vivo *events*

Despite the fact that HD is a disease of selective neuronal death, this 293T *in vitro* model (Martindale *et al.* 1998) has previously been shown to recapitulate several features of HD. As observed *in vivo*, mutant huntingtin and other disease proteins containing expanded polyglutamine tracts form aggregates *in vitro*, whereas wild-type proteins with a normal tract do not (Lunkes & Mandel 1998; Igarashi *et al.* 1998; Skinner *et al.* 1997; Ellerby *et al.* 1999*a*; Hackam *et al.* 1998*a*; Cooper *et al.* 1998; Butler *et al.* 1998; Merry *et al.* 1998; Li & Li 1998). Aggregates formed *in vivo* and *in vitro* are frequently ubiquitinated (Lunkes & Mandel 1998; Cooper *et al.* 1998; Igarashi *et al.* 1998; Cummings *et al.* 1998; A. S. Hackam, unpublished observations). Furthermore, as observed in post-mortem neocortical tissue (Becher *et al.* 1998), increasing polyglutamine length is associated with an increased frequency of aggregates *in vitro* (Martindale *et al.* 1998; Li & Li 1998; Lunkes & Mandel 1998). More severe grades of HD have a higher frequency of cortical nuclear inclusions (Becher *et al.* 1998). Similarly, huntingtin fragments that are more toxic *in vitro* form aggregates with higher frequency (Hackam *et al.* 1998*a*; Cooper *et al.* 1998). The formation of huntingtin cleavage fragments is observed both *in vivo* and in cell culture (DiFiglia *et al.* 1997; Martindale *et al.* 1998; Lunkes & Mandel 1998). Finally, the selective vulnerability of affected neurons in HD is mimicked by increased susceptibility to apoptotic stress of cultured cells expressing mutant huntingtin (Hackam *et al.* 1998*a*; Cooper *et al.* 1998; Martindale *et al.* 1998). Therefore, it is plausible that these *in vitro* data, which recapitulate the *in vivo* situation to a significant extent, are relevant, suggesting that nuclear and cytoplasmic aggregates may also have equivalent toxicity in humans.

(c) *Aggregates are not the sole contributors to toxicity*

Intracellular aggregates, regardless of their cellular compartment, are associated with toxicity. Therefore, reducing aggregate frequency remains an important therapeutic target for CAG expansion diseases. At the present time, it is unclear whether aggregates are the primary cause of neurodegeneration, or whether they are formed as an early secondary response to cell injury. The observation that the development of aggregates precedes cell death *in vivo* and *in vitro* does not distinguish between aggregate formation as a causal event, or aggregates as a by-product of other cytotoxic events that lead to death. Aggregates could theoretically even serve a protective role by sequestering toxic polyglutamine-containing fragments.

There is increasing evidence that aggregates are not the sole contributors to toxicity. Although the majority of inclusions have been identified in neuronal populations that degenerate during disease progression, several studies have shown that the concordance between nuclear inclusions and neurodegeneration is not absolute (Becher *et al.* 1998; Hodgson *et al.* 1999; Holmberg *et al.* 1998; Li *et al.* 1998; Warrick *et al.* 1998). There are several examples in which inclusions have been identified in cells not destined to die. Aggregates were present in the dentate nucleus of the cerebellum in HD patients (Becher *et al.* 1998), an area that does not frequently exhibit neurodegeneration. Intranuclear inclusions were also identified in SCA-7 (Holmberg *et al.* 1998) and DRPLA (Becher *et al.* 1998) patients in regions of the brain not affected by the disease. Ubiquitinated intranuclear inclusions formed by mutant androgen receptor aggregates were observed in SMBA patients in peripheral tissues (Li *et al.* 1998). In addition, mice transgenic for mutant huntingtin exon 1 (Davies *et al.* 1997) and a *Drosophila* model of SCA-3 (Warrick *et al.* 1998), contained aggregates in regions that do not exhibit cell death. Finally, the presence of selective medium spiny degeneration in the absence of macro- or micro-aggregates clearly indicates that aggregates are not essential for initiation of cell death (Hodgson *et al.* 1999). These observations suggest that aggregate formation is associated, but is clearly not necessary to cause cell death.

There are also reports in which nuclear inclusions were not observed in affected tissues. Neurodegeneration of Purkinje cells in SCA-7 and juvenile HD patients occurred in the absence of aggregates (Holmberg *et al.* 1998; Becher *et al.* 1998). Further, there is no apparent correlation of nuclear inclusion frequency with length of the CAG tract and Vonsattel grade in the striatum of HD patients (Becher *et al.* 1998).

These observations argue against a role of aggregates as a direct cause of neurodegeneration. However, aggregates may result in deficiencies in neuronal function

(Davies *et al.* 1997; Lunkes & Mandel 1997; Ross 1997) and additional events may be required to lead to neurodegeneration subsequent to the formation of aggregates. For example, although neurons may initially form aggregates as a result of stress-induced protein cleavage, a particular cellular environment may be required for cytotoxicity, analogous to the tamoxifen-induced stress in our 293T cell model. In neurons, this 'toxic environment', possibly resulting from expression of certain glutamate receptors leading to uncontrolled excitotoxicity, may be the stimulus needed for aggregate-containing cells to die. The cells in non-affected tissues that form aggregates could be more resistant to toxicity if they have a higher threshold of injury needed for death. The threshold could be set by the particular repertoire of glutamate receptors, by the cell's ability to deal with metabolic stress, or by levels of anti-apoptotic factors. Thus, aggregates alone are insufficient for cell death, but selective populations of vulnerable neurons may be more susceptible to a 'toxic environment' when their viability is compromised by the presence of aggregates.

(d) *Huntingtin is toxic in both the nucleus and cytoplasm of 293T cells*

Determining the primary site of pathology of the CAG diseases is important for designing therapeutic interventions. Nuclear inclusions are associated with disease in the other CAG diseases studied so far (Skinner *et al.* 1997), whereas for HD both nuclear and extranuclear aggregates are seen *in vivo* and *in vitro* (DiFiglia *et al.* 1997).

The different localization of aggregates in HD compared with other expansion diseases may depend on several factors. First, the size of a protein influences nuclear entry (Görlich & Mattaj 1996). Thus, the ability of the protein to be cleaved into fragments small enough to enter the nucleus is important. Ataxin-3 is small enough to diffuse into the nucleus. The mutant exon 1 fragment in the HD mice forms nuclear inclusions since it is also small enough to diffuse into the nucleus. Only antibodies against N-terminal epitopes recognize nuclear inclusions in HD post-mortem tissue, suggesting proteolytic processing of full-length huntingtin (DiFiglia *et al.* 1997; Becher *et al.* 1998).

Second, the polyglutamine-containing proteins may usually reside in the nucleus as part of their normal function. For example, ataxin-1 (Skinner *et al.* 1997) is predominantly normally localized in the nucleus of neurons affected in SCA-1. Thus, nuclear entry is not a feature of this disease. In addition, ataxin-3 (Tait *et al.* 1998; Paulson *et al.* 1997), ataxin-7 (Stevanin *et al.* 1996; Trottier *et al.* 1995), the androgen receptor (Li *et al.* 1998) and atrophin-1 (Miyashita *et al.* 1997) have putative nuclear localization sequences and have been identified in the nucleus. By contrast, we have not found an active NLS in the N-terminus of huntingtin. Third, there may be inherent differences in the pathogenesis of these diseases due to differences in functional properties or protein partners of the respective proteins. For example, androgen receptor toxicity is influenced by ligand concentration in 293T cells (Ellerby *et al.* 1999*a*), and huntingtin associates with several proteins that have altered interactions with increased polyglutamine length (Li *et al.* 1995; Burke *et al.* 1996; Kalchman *et al.* 1997). Ataxin-1 and ataxin-3 associate with the nuclear matrix (Matilla *et al.* 1997; Tait *et al.* 1998) and may interfere with essential nuclear events as part of their toxicity. Indeed, recent results from ataxin-1 transgenic mouse lines by Klement *et al.* (1998) indicate that nuclear localization of ataxin-1 is critical for SCA-1 pathology. Finally, detection of the localization of a particular protein could vary with tissue preparation and the antisera used.

There is clearly *in vivo* evidence for extranuclear aggregates being toxic (DiFiglia *et al.* 1997). While this paper was under review Saudou *et al.* (1998) presented data that indicated that nuclear localization was required for cytotoxicity of huntingtin in a striatal neuronal line. There are several methodological differences that could account for the discordant conclusions between the present paper and the work of Saudou *et al.* In this study, we have assessed the total frequency of aggregates, including cytoplasmic aggregates, which allowed direct comparison of the influence of total cellular aggregates on toxicity in 293T cells. Additionally, there are differences in sensitivities and timing of the cell viability assays used. The MTT assay quantifies mitochondrial changes, which are considered as earlier indicators of apoptosis than nuclear morphological changes (Green & Reed 1998). Mitochondrial markers of apoptosis may record early subtle alterations of cell viability caused by cytoplasmic huntingtin. In addition, further studies are needed to determine whether cell line-specific factors may contribute to the differences in these findings.

(e) *YAC transgenic mice expressing mutant huntingtin have cytoplasmic and nuclear changes*

We have produced yeast artificial chromosome (YAC) transgenic mice expressing normal and mutant huntingtin that is expressed in a developmentally regulated and cell-specific manner essentially identical to that seen with endogenous huntingtin. The YAC transgenic mice expressing mutant huntingtin with 46 repeats do not have a clinical phenotype as evidenced by detailed neurological and behavioural assessment up until the latest stage of assessment at 24 months. However, mild electrophysiological abnormalities do become evident in these mice at six months of age. These abnormalities become more obvious and are readily apparent at ten months of age when diminished hippocampal long-term potentiation in the CA1 neurons is evident. This is also associated with an increase in intracellular calcium stores. At ten months of age detailed assessment using electron microsopy and immunogold labelling has failed to reveal any evidence for nuclear translocation of huntingtin aggregates or evidence for neurodegeneration. However, further examination of these mice at approximately two years of age has revealed evidence for labelling of N-terminal huntingtin, which is seen traversing the nuclear pore, and also seen in the nucleus of selected neurons. This indicates that nuclear translocation of huntingtin occurs after some cellular abnormalities are apparent in these mice. Furthermore, these mice have no evidence for a clinical phenotype, also indicating that nuclear translocation of huntingtin occurs prior to evidence for a clinical phenotype.

Mice expressing huntingtin with 72 glutamines at levels less than endogenous levels showed a clinical

phenotype at nine months. Detailed pathological examination has revealed evidence for increased cellular staining and medium spiny neuronal loss in the lateral striatum. The examination of a founder mouse with 72 repeats that had increased copy number of the YACs with evidence for increased expression, had an obvious clinical phenotype by six weeks of age and when examined pathologically at one year of age had obvious evidence for proteolytic processing of huntingtin with some N-terminal labelled huntingtin present in the cytoplasm, some labelling being seen in the nuclear pores, and other neurons with clear evidence of significant seeding into the nucleus of this huntingtin fragment. These N-terminal huntingtin fragments either coalesced with a few N-terminal huntingtin fragments interacting with each other, and then in some cells this was associated with evidence for large numbers of N-terminal fragments coalescing into microaggregates, and in other cells with a development of obvious aggregates seen at light microscopy (Hodgson *et al*. 1999).

These studies give some indication of the process for the development of aggregates with initial proteolytic processing of huntingtin in the cytoplasm, liberation of an N-terminal fragment which traverses the nuclear pore and enters into the nucleus. Within the nucleus there is initial seeding of huntingtin with other N-terminal huntingtin fragments over time, and as the protein concentration within the nucleus of internal huntingtin increases, this allows N-terminal huntingtin fragments to interact with each other with eventual production of aggregates associated with neurodegeneration.

The YAC transgenic mice expressing 46 repeats demonstrated some cytoplasmic toxicity with increased calcium concentration and obvious electrophysiological abnormalities. The earlier presentation of a neurological phenotype associated with neurodegeneration by one year of age of the YAC-72 mice is compatible with the accelerated disease seen in patients who manifest with juvenile HD associated with polyglutamine expansion in a similar repeat range. Polyglutamine expansion of this range is associated with presentation by ten years of age and clearly is compatible with a highly accelerated disease process. In juvenile onset, the cytoplasmic phase of this illness may be shorter, associated with the accelerated development of intranuclear aggregates occurring at a much earlier stage than seen, for example, in the mice with 46 repeats when N-terminal translocation of huntingtin was only first seen by two years of age. This would suggest that in patients with 46 repeats the cytoplasmic phase of the illness with some evidence for toxicity may occur for a longer period and that only much closer to, but prior to, onset of the clinical illness would this be associated with nuclear translocation of huntingtin as seen in the YAC transgenic mice expressing 46 glutamines. These data provide further *in vivo* evidence for the nucleus and cytoplasm as sites of pathogenesis in HD.

We are grateful to Dr Gideon Dreyfuss for use of the pyruvate kinase construct and for helpful discussion. We also thank Krista McCutcheon and Keith Fichter for their excellent technical expertise, and Dr Cheryl Wellington and the entire HD laboratory group for useful discussions. This work is supported by the Canadian Network Centres of Excellence (NCE, Genetics) and the Medical Research Council (MRC, Canada). A.S.H. is an MRC post-doctoral fellow. M.R.H. is an established investigator of the British Columbia Children's Hospital. The *in vitro* work reported here is also part of an original publication in *Human Molecular Genetics* (Hackam *et al*. 1999).

REFERENCES

Andrew, S. E., Goldberg, Y. P. & Hayden, M. R. 1997 Commentary: rethinking genotype and phenotype correlations in polyglutamine expansion disorders. *Hum. Mol. Genet.* **6**, 2005–2010.

Becher, M. W., Kotzuk, J. A., Sharp, A. H., Davies, S. W., Bates, G. P., Price, D. L. & Ross, C. A. 1998 Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and palidoluysian atrophy: correlation between the density of inclusions and *IT15* CAG triplet repeat length. *Neurobiol. Dis.* **4**, 387–397.

Bots, G. T. & Bruyn, G. W. 1981 Neuropathological changes of the nucleus accumbens in Huntington's chorea. *Acta Neuropathol.* **55**, 21–22.

Burke, J. R., Enghild, J. J., Martin, M. E., Jou, Y. S., Myers, R. M., Roses, A. D., Vance, J. M. & Strittmatter, W. J. 1996 Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH. *Nature Med.* **2**, 347–350.

Butler, R., Leigh, P. N., McPhaul, M. J. & Gallo, J.-M. 1998 Truncated forms of the androgen receptor are associated with polyglutamine expansion in X-linked spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* **7**, 121–127.

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. & Mitchell, J. B. 1987 Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **47**, 936–942.

Cooper, J. K. (and 12 others) 1998 Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture. *Hum. Mol. Genet.* **7**, 783–790.

Cummings, C. J., Mancini, M. A., Antalffy, B., DeFranco, D. B., Orr, H. T. & Zoghbi, H. Y. 1998 Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nature Genet.* **19**, 148–154.

Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L. & Bates, G. P. 1997 Formation of neuronal intranuclear inclusions (NII) underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell* **90**, 537–548.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P. & Aronin, N. 1997 Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* **277**, 1990–1993.

Ellerby, L. M. (and 10 others) 1999*a* Kennedy's disease: caspase cleavage of the androgen receptor is a crucial event in cytotoxicity. *J. Neurochem.* **72**, 185–195.

Ellerby, L. M. (and 11 others) 1999*b* Cleavage of atrophin-1 at caspase site aspartic acid 109 modulates cytotoxicity. *J. Cell Biol.* **274**, 8730–8736.

Goldberg, Y. P. (and 10 others) 1996 Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nature Genet.* **13**, 442–449.

Gourfinkel-An, I., Cancel, G., Duyckaerts, C., Faucheux, B., Hauw, J.-J., Trottier, Y., Brice, A., Agid, Y. & Hirsch, E. C. 1998 Neuronal distribution of intranuclear inclusions in Huntington's disease with adult onset. *NeuroReport* **9**, 1823–1826.

Görlich, D. & Mattaj, I. W. 1996 Nucleocytoplasmic transport. *Science* **271**, 1513–1518.

Green, D. R. & Reed, J. C. 1998 Mitochondria and apoptosis. *Science* **281**, 1309–1312.

Hackam, A. S., Singaraja, R., Wellington, C. L., Metzler, M., McCutcheon, K., Zhang, T., Kalchman, M. & Hayden,

M. R. 1998*a* The influence of huntingtin protein size on nuclear localization and cellular toxicity. *J. Cell Biol.* **141**, 1097–1105.

Hackam, A. S., Wellington, C. L. & Hayden, M. R. 1998*b* The fatal attraction of polyglutamine-containing proteins. *Clin. Genet.* **53**, 233–242.

Hackam, A. S., Singaraja, R., Zhang, T., Gan, L. & Hayden, M. R. 1999 *In vitro* evidence for both the nucleus and cytoplasm as subcellular sites of pathogenesis in Huntington disease. *Hum. Mol. Genet.* **8**, 25–33.

Hodgson, J. G. (and 18 others) 1999 A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity and striatal neurodegeneration. *Neuron*. (In the press.)

Holmberg, M. (and 10 others) 1998 Spinocerebellar ataxia type 7 (SCA7): a neurodegenerative disorder with neuronal intranuclear inclusions. *Hum. Mol. Genet.* **7**, 913–918.

Igarashi, S. (and 18 others) 1998 Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. *Nature Genet.* **18**, 111–117.

Kalchman, M. A. (and 13 others) 1997 HIPl, a human homolog of *S. cerevisiae* Sla2p, interacts with membrane-associated huntingtin in the brain. *Nature Genet.* **16**, 44–53.

Klement, I. A., Skinner, P. J., Kaytor, M. D., Yi, H., Hersch, S. M., Clark, H. B., Zoghbi, H. Y. & Orr, H. T. 1998 Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in *SCA1* transgenic mice. *Cell* **95**, 41–53.

Li, M., Nakagomi, Y., Kobayashi, Y., Merry, D. E., Tanaka, F., Doyu, M., Mitsuma, T., Hashizume, Y., Fischbeck, K. H. & Sobue, G. 1998 Non-neural inclusions of androgen receptor protein in spinal and bulbar muscular atrophy. *Am. J. Pathol.* **153**, 695–701.

Li, S.-H. & Li, X.-J. 1998 Aggregation of N-terminal huntingtin is dependent on the length of its glutamine repeats. *Hum. Mol. Genet.* **7**, 777–782.

Li, X. J., Li, S. H., Sharp, A. H., Nucifora Jr, F. C., Schilling, G., Lanahan, A., Worley, P., Snyder, S. H. & Ross, C. A. 1995 A huntingtin-associated protein enriched in brain with implications for pathology. *Nature* **378**, 398–402.

Lunkes, A. & Mandel, J.-L. 1997 Polyglutamines, nuclear inclusions and neurodegeneration. *Nature Med.* **8**, 1201–1202.

Lunkes, A. & Mandel, J.-L. 1998 A cellular model that recapitulates major pathogenic steps of Huntington's disease. *Hum. Mol. Genet.* **7**, 1355–1361.

Martindale, D. (and 11 others) 1998 Length of the protein and polyglutamine tract influence localization and frequency of intracellular aggregates of huntingtin. *Nature Genet.* **18**, 150–154.

Matilla, A., Koshy, B. T., Cummings, C. J., Isobe, T., Orr, H. T. & Zoghbi, H. Y. 1997 The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1. *Nature* **389**, 974–978.

Merry, D. E., Kobayashi, Y., Bailey, C. K., Taye, A. A. & Fischbeck, K. H. 1998 Cleavage, aggregation and toxicity of the expanded androgen receptor in spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* **7**, 693–701.

Miyashita, T., Okamura-Oho, Y., Mito, Y., Nagafuchi, S. & Yamada, M. 1997 Dentatorubral pallidoluysian atrophy (DRPLA) protein is cleaved by caspase-3 during apoptosis. *J. Biol. Chem.* **272**, 29 238–29 242.

Ordway, J. M. (and 11 others) 1997 Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. *Cell* **91**, 753–763.

Paulson, H. L., Perez, M. K., Trottier, Y., Trojanowski, J. Q., Subramony, S. H., Das, S. S., Vig, P., Mandel, J.-L., Fischbeck, K. H. & Pittman, R. N. 1997 Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron* **19**, 333–344.

Ross, C. 1997 Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? *Neuron* **19**, 1147–1150.

Sapp, E., Schwarz, C., Chase, K., Bhide, P. G., Young, A. B., Penney, J., Vonsattel, J. P., Aronin, N. & DiFiglia, M. 1997 Huntingtin localization in brains of normal and Huntington's disease patients. *Ann. Neurol.* **42**, 604–612.

Saudou, F., Finkbeiner, S., Devys, D. & Greenberg, M. E. 1998 Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* **95**, 55–66.

Skinner, P. J., Koshy, B. T., Cummings, C. J., Klement, I. A., Helin, K., Servadio, A., Zoghbi, H. Y. & Orr, H. T. 1997 Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures. *Nature* **389**, 971–974.

Stevanin, G. (and 17 others) 1996 Screening for proteins with polyglutamine expansions in autosomal dominant cerebellar ataxias. *Hum. Mol. Genet.* **5**, 1887–1892.

Tait, D., Riccio, M., Sittler, A., Scherzinger, E., Santi, S., Ognibene, A., Maraldi, N. M., Lehrach, H. & Wanker, E. E. 1998 Ataxin-3 is transported into the nucleus and associates with the nuclear matrix. *Hum. Mol. Genet.* **7**, 991–997.

Tellez-Nagel, I., Johnson, A. B. & Terry, R. D. 1974 Studies on brain biopsies of patients with Huntington's chorea. *J. Neuropathol. Exp. Neurol.* **33**, 308–332.

Trottier, Y. (and 12 others) 1995 Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. *Nature* **378**, 403–406.

Warrick, J. M., Paulson, H. L., Gladys, L., Gray-Board, G. L., Bui, Q. T., Fischbeck, K. H., Pittman, R. N. & Bonini, N. M. 1998 Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell* **93**, 939–949.

Wellington, C. L. (and 20 others) 1998 Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J. Biol. Chem.* **273**, 9159–9167.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES