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Transgenic models of Huntington's disease

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Huntington's disease (HD) is an inherited neurodegenerative disorder caused by a CAG–polyglutamine repeat expansion. A mouse model of this disease has been generated by the introduction of exon 1 of the human HD gene carrying highly expanded CAG repeats into the mouse germ line (R6 lines). Transgenic mice develop a progressive neurological phenotype with a movement disorder and weight loss similar to that in HD. We have previously identified neuronal inclusions in the brains of these mice that have subsequently been established as the pathological hallmark of polyglutamine disease. Inclusions are present before symptoms, which in turn occur long before any selective neuronal cell death can be identified. We have extended the search for inclusions to skeletal muscle, which, like brain, contains terminally differentiated cells. We have conducted an investigation into the skeletal muscle atrophy that occurs in the R6 lines, (i) to provide possible insights into the muscle bulk loss observed in HD patients, and (ii) to conduct a parallel analysis into the consequence of inclusion formation to that being performed in brain. The identification of inclusions in skeletal muscle might be additionally useful in monitoring the ability of drugs to prevent inclusion formation *in vivo*.

Keywords: Huntington's disease; polyglutamine repeat; transgenic mouse; neurodegeneration; amyloid

1. THE POLYGLUTAMINE NEURODEGENERATIVE DISEASES

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease. Although onset is generally within the fourth or fifth decade, the disease can start at any time from early childhood until very old age with a mean duration of 15–20 years (Harper 1996). The inheritance pattern of HD shows anticipation when transmitted through the male line in that most early-onset cases inherit the mutation from their fathers (Farrer *et al*. 1992). Patients exhibit a diverse set of symptoms, with well-recognized emotional, cognitive and motor components. The movement disorder can seem drastically different between the adult and early-onset forms of the disease in that the juvenile patients can exhibit Parkinsonian-like features and never express chorea. Neuropathological examination shows that most specific cell loss occurs in the cortex and the striatum (Myers *et al*. 1991; Vonsattel *et al*. 1985), although this can be more widespread in the juvenile disease, frequently including the Purkinje cells of the cerebellum (Young 1998). A more general reduction in brain size also occurs across all regions (de la Monte *et al*. 1988). In addition, HD patients need a high calorific intake and usually find difficulty in maintaining their body weight, with a marked loss in muscle bulk (Harper 1996; Sanberg *et al*. 1981).

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The HD gene contains 67 exons and extends across 170 kb of DNA (Ambrose *et al*. 1993; Baxendale *et al*. 1995). The CAG repeat that is expanded on HD chromosomes lies within exon 1 and is translated into a stretch of polyglutamine (polyQ) residues. The normal and expanded ranges are $(CAG)_{6–39}$ and $(CAG)_{36–180}$, respectively (Rubinsztein *et al*. 1996; Sathasivam *et al*. 1997; Stine *et al*. 1993). Most adult-onset cases have expansions ranging from 40 to 55 units, whereas expansions of 70 and above invariably cause the juvenile form of the disease. The normal and mutant forms of huntingtin have been shown to be expressed at similar levels in the central nervous system (CNS) and in peripheral tissues (Trottier *et al*. 1995). Within the brain, huntingtin was found predominantly in neurons and was present in cell bodies, dendrites and also in the nerve terminals. Immunohistochemistry, electron microscopy and subcellular fractionations have shown that huntingtin is primarily a cytosolic protein associated with vesicles and/or microtubules, suggesting that it has a functional role in cytoskeletal anchoring or transport of vesicles (DiFiglia *et al*. 1995; Gutekunst *et al*. 1995; Sharp *et al*. 1995). Huntingtin has also been detected in the nucleus (de Rooij *et al*. 1996) in mouse embryonic fibroblast, adult fibroblast and neuroblastoma cell lines.

In addition to HD, a polyQ expansion has been found to cause seven other late-onset inherited neurodegenerative diseases, namely spinal and bulbar muscular atrophy (SBMA), dentatorubral–pallidoluysian atrophy (DRPLA)

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Table 1. *Summary of the exon 1 huntingtin transgenic mouse lines*

(Reproduced from Bates *et al*. (1998), with the permission of the International Society of Neuropathology.)

transgenic line	integration site	CAG repeat size	transgene expression	phenotype
R6/1	single copy	113	+	+
R6/2	one intact copy	144	+	+
R6/5	four intact copies	128–156	+	+
R6/0	single copy	142	–	–
HDex6	*ca*. 20 copies	18	+	–
HDex27	*ca*. seven copies	18	+	–

and the spinocerebellar ataxias (SCA) 1, 2, 3, 6 and 7 (see references in Bates *et al*. (1998)). These diseases bear many similarities: they are autosomal-dominant (with the exception of X-linked SBMA, the dominant nature of which is probably masked by X-inactivation), often show anticipation and have broadly comparable normal and expanded repeat ranges. With the possible exception of SCA6 (which has a smaller expanded repeat range), it is expected that these diseases operate via the same molecular mechanism. The molecular event that triggers pathogenesis must correlate with the polyQ pathogenic size threshold and account for the late onset of the disorders. The proteins that harbour the polyQ tracts are otherwise unrelated and have widely overlapping expression patterns. Therefore the basis for the differential selective neuronal vulnerability between these diseases must also be explained (Ross 1995; Young 1998).

2. THE R6 TRANSGENIC LINES

The demonstration by gene targeting that nullizygosity for the mouse HD gene (*hd*) is embryonic lethal suggested that the HD mutation is more likely to act via a 'gain of function' rather than a 'dominant-negative' mechanism (Duyao *et al*. 1995; Nasir *et al*. 1995; Zeitlin *et al*. 1995). The introduction of the HD mutation into the mouse germ line could therefore be predicted to generate a mouse model of HD irrespective of the presence of two copies of *hd*. The R6 transgenic lines contain a 2 kb genomic fragment that spans the 5′ end of the human gene, encompasses 1 kb of control elements and generates an N-terminal exon 1 protein corresponding to *ca*. 3% of huntingtin (Mangiarini *et al*. 1996). Six lines have been established, the main features of which are summarized in table 1. Four lines contain expanded CAG repeats of a size larger than that generally associated with the juvenile form of the disease, namely R6/1 $(CAG)_{115}$, R6/2 $(CAG)_{145}$, R6/5 $(CAG)_{135–156}$ and R6/0 $(CAG)_{142}$ (Mangiarini *et al*. 1997); a further two lines, HDex6 and HDex27, contained $(CAG)_{18}$ as normal repeat controls. In all cases except line R6/0, in which the transgene is probably silenced by the site of integration, the transgene protein showed a ubiquitous tissue expression profile.

A progressive neurological phenotype develops in lines R6/1, R6/2 and R6/5 in which the CAG repeats are expanded and the transgene is expressed. On the basis of home cage behaviour, the onset ages are approximately two months in line R6/2 and 4–5 months in line R6/1. The movement disorder includes an irregular gait, stereotypic grooming movements, rapid shudders (similar to a wet dog shake), a tremor (or very rapid myoclonus) and a tendency to clasp the hindlimbs and forelimbs together when suspended by the tail. These movements are not associated with seizure activity (J. Noebels, unpublished data). In addition, a proportion of the mice develop tonic–clonic seizures. A detailed analysis of motor function as measured by performance on the rotarod, beam walking, swimming and footprint analysis shows that the age of onset differs depending on the nature and difficulty of the task being measured (Carter *et al*. 1999), and that significant differences between the R6/2 transgenic animals and their littermate controls can be detected as early as at five weeks. In addition to the movement disorder, the mice exhibit a progressive weight loss. The phenotype progresses rapidly and mice are rarely kept beyond 12 weeks of age, by which time the movement disorder is pronounced and the transgenic animals weigh between 60% and 70% of their littermate controls. A small number of mice have been studied to 16–17 weeks of age.

3. POLYGLUTAMINE AGGREGATION AND NEURONAL INCLUSIONS

A neuropathological analysis of serial sections throughout the entire brain and spinal cord from R6/2 mice at 12 weeks of age revealed no evidence of selective neuronal cell death (Mangiarini *et al*. 1996). Transgenic animal brains were consistently smaller than those from their littermate controls, a size reduction that began after four weeks and resulted in a loss of brain weight of *ca*. 20% by 12 weeks of age. However, immunohistochemistry with antibodies directed against the N-terminus of the huntingtin protein identified neuronal intranuclear inclusions (NII) as an intense focus of reaction product in the nucleus (Davies *et al*. 1997). These inclusions could be identified at the ultrastructural level, in the absence of immunostaining, as a granular and fibrillar structure devoid of a membrane and slightly larger than the nucleolus. They could be first identified before four weeks in the cerebral cortex. However, neurodegeneration is not present in R6/2 brains until considerably later than inclusions can be detected. The first appearance of condensing neurons occurs in the anterior cingulate cortex at 14 weeks of age and progresses in this brain region until 16–17 weeks—the latest time point at which it has been possible for us to study these mice (Davies *et al*., this issue).

To gain insight into the molecular interactions that underlie the pathogenesis in the R6 lines, various huntingtin exon 1 proteins containing polyQ repeats ranging from 20Q to 122Q have been expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* (Scherzinger *et al*. 1997; B. Hollenbach *et al*., this issue). After purification, proteins containing repeats in the pathogenic range spontaneously formed ordered aggregates, as in 83Q and 122Q, or after removal of the GST tag in 51Q. When stained with Congo red and viewed under polarized light, these aggregates showed a green colour and birefringence indicative of amyloid (Scherzinger *et al*. 1997). This is consistent with the prediction of Max Perutz that polyQ tracts are capable of interacting via hydrogen bonding between main-chain and side-chain amides in a cross-β-sheet structure, an interaction that he termed a 'polar zipper' (Perutz *et al*. 1994). A more recent detailed analysis of the polyQ length and concentration dependence of polyQ aggregation in the context of huntingtin has shown that the ability to form ordered aggregates corresponds to the pathogenic threshold observed in the disease (Scherzinger *et al*. 1999).

The symptoms observed in the R6 lines occur after the appearance of neuronal inclusions but long before a specific cell loss can be detected. Insights into a possible cause of this neuronal dysfunction have arisen from neurotransmitter receptor binding and expression studies (Cha *et al*. 1998). To explore the relationship between glutamate, and other receptors known to be affected in HD, and the symptoms in R6/2 mice, receptors were examined in the brains of mice aged 4, 8 and 12 weeks by using receptor-binding autoradiography, immunoblotting for receptor proteins and hybridization *in situ*. At 12 weeks, hybridization *in situ* showed marked decreases in mRNA expression for mGluR1, mGluR2 and mGluR3 metabotropic receptors and D_1 and D_2 dopamine receptors. The decreases in mGluR1, D_1 and D_2 dopamine receptor mRNA signals in the striatum were statistically significant by four weeks of age and the decrease in mGluR2 mRNA expression in the cortex was significant by eight weeks of age. Therefore by eight weeks there are major alterations in the dopamine and glutamate neurotransmitter systems, both of which are important in striatal function (Cha *et al*. 1998; B. Hollenbach *et al*., this issue). The presence of the mutant HD protein in the nucleus, possibly as a conformational intermediate or in the early stages of aggregation, might alter the expression of multiple genes, including those encoding specific neurotransmitter receptors (Cha *et al*. 1998).

4. IDENTIFICATION OF INCLUSIONS IN SKELETAL MUSCLE

Within the R6 mouse brains, inclusions are readily identifiable in neurons but are seen only rarely in glial cells. The terminally differentiated nature of neurons might allow time for the critical concentration of the precursor for aggregation to be achieved. This would be consistent with the nucleation and aggregation model of aggregate formation that has been proposed for other amyloidogenic diseases (Lansbury 1997). The terminally differentiated nature of neurons might therefore explain why, despite the wide expression profiles of the various polyQ-containing proteins, polyQ expansion results in neurodegenerative disease. This interpretation is also supported by the ectopic expression of a polyQ expansion in the mouse *hprt* gene resulting in neuronal intranuclear inclusions and a neurological phenotype (Ordway *et al*. 1997).

If the formation of polyQ aggregates and ultimately inclusions is a concentration-dependent process, it might be expected that these structures could form in other terminally differentiated cells outside the CNS. To explore this possibility, immunohistochemistry was performed to determine whether polyQ inclusions are present in skeletal muscle. Frozen sections were prepared in both the transverse and longitudinal plains from R6/2 quadricep at 14 weeks of age and from R6/1 quadricep at 15 months. Inclusions were identified with antibodies both against the N-terminus of huntingtin and against ubiquitin (figure 1). They were absent at four weeks but present by six weeks. Inclusions were always nuclear and an apparent nuclear association was probably due to the distortion of the nucleoplasm during the section preparation. Under light microscopy there often seemed to be more than one inclusion per nucleus, although it was clear from the electron microscopic analysis that in at least some cases this was due to a single inclusion in each of two adjacent nuclei. At the ultrastructural level, the inclusions seemed identical with those previously described in neurons (figure 2).

5. AN ANALYSIS OF MUSCLE ATROPHY OCCURRING IN THE R6 LINES

HD patients have a high calorific requirement; a loss of skeletal muscle bulk is a well-described feature of the disease (Harper 1996; Sanberg *et al*. 1981). Similarly, a progressive decrease in skeletal muscle bulk occurs in the R6 lines. The onset of muscle atrophy begins after six weeks, and by 12 weeks the quadricep and forecalf muscles have decreased to 42% and 47%, respectively, of the mass of these muscles in the non-transgenic littermates (Sathasivam *et al*. 1999).

To gain further insights into the consequence of inclusion formation and into the cause of the muscle atrophy, we have conducted a histological analysis of skeletal muscle from 14-week-old R6/2 and 15-month-old R6/1 mice. Transverse sections were cut from snap-frozen tissue and stained with haematoxylin and eosin (H and E), haematoxylin–van Gieson (HVG) and the periodic acid–Schiff (PAS) assay. Acid phosphatase and NADH–tetrazolium reductase (NADH–TR) enzyme activities were measured and immunohistochemistry was performed with the anti-N-cam antibody (H28, Boehringer Mannheim, 1:500 dilution).

There was little evidence of a myopathy as indicated by muscle fibre regeneration. The H and E staining showed no evidence of an appreciable increase in the frequency of internal nuclei (figure 3). Similarly, vesicular nuclei or fibre splitting could not be detected and N-cam immunoreactivity did not show an increased frequency of regenerating fibres (Sathasivam *et al*. 1999). There was no evidence of a focal atrophy or abnormal distribution of motor endplates, suggesting that the atrophy is not caused

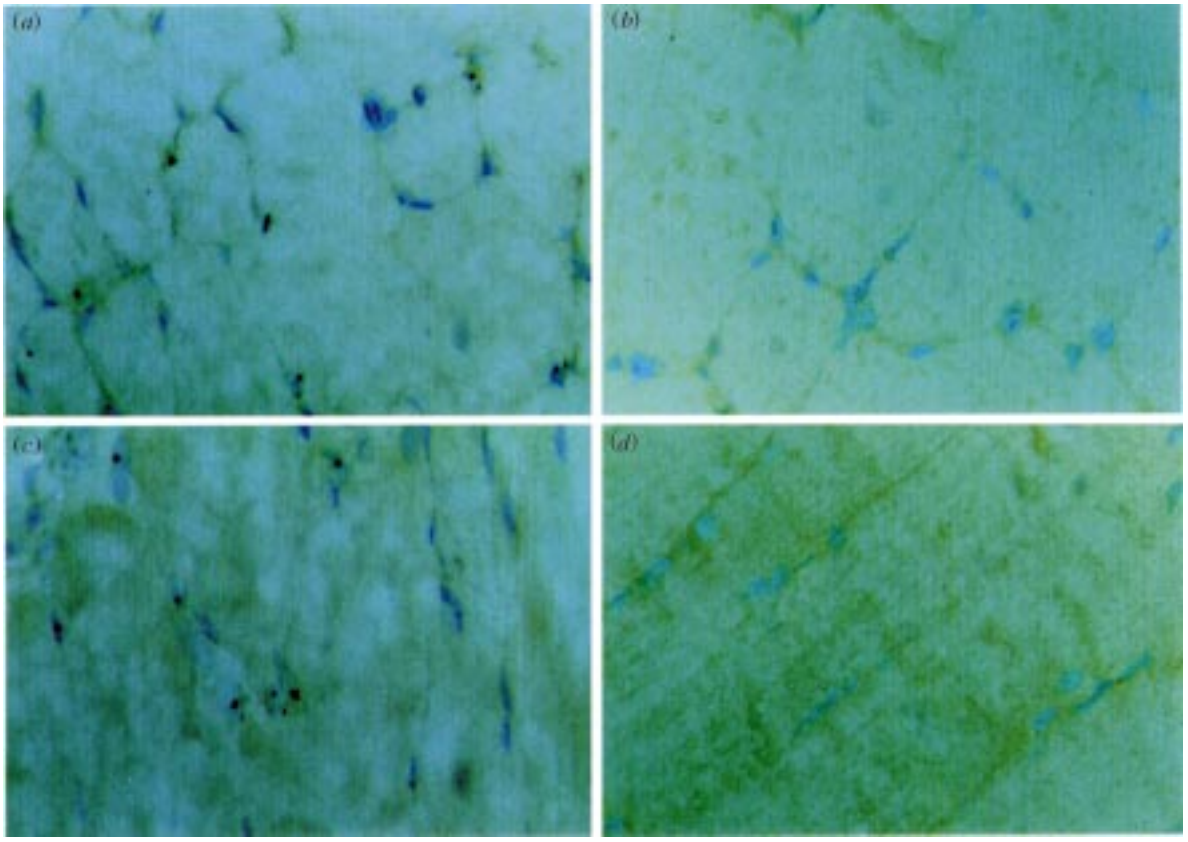

Figure 1. Nuclear inclusions in skeletal muscle from the R6 lines. Snap-frozen sections were cut from the quadricep muscle in transverse (*a*, *b*) and longitudinal (*c*, *d*) planes and immunostained with an anti-ubiquitin (Dako). (*a*) Fifteen-month-old R6/1 transgenic animal; (*b*) 15-month-old non-transgenic control; (*c*) eight-week-old R6/2 transgenic animal; (*d*) eight-week-old non-transgenic control.

by denervation. Acid phosphatase activity showed no evidence of cellular infiltration in response to necrotic fibres (figure 3), and granular or basophilic fibres were not apparent on the H and E stained sections. The HVG stained sections showed no fibrosis as evident from the proliferation of the endomysial or perimysial connective tissue (Sathasivam *et al*. 1999). The PAS staining, NADH–TR enzyme reactions and immunohistochemistry with antibodies against myosin heavy chain type 1, type 2 and type 2A skeletal muscle fibres showed no evidence of atrophy of a specific fibre type (Sathasivam *et al*. 1999). This was supported by a morphometric analysis that indicated that a uniform shrinkage across all muscle fibre types had occurred (Sathasivam *et al*. 1999).

Ultrastructural analysis of R6/2 quadricep muscle at 14 weeks showed that the only difference between muscle sections from the transgenic animals and the littermate controls was the presence of nuclear inclusions. However, Although no gross differences were apparent in the analysis described above, non-specific ultrastructural degenerative changes could be identified in the R6/1 transgenic animal aged 15 months (Sathasivam *et al*. 1999).

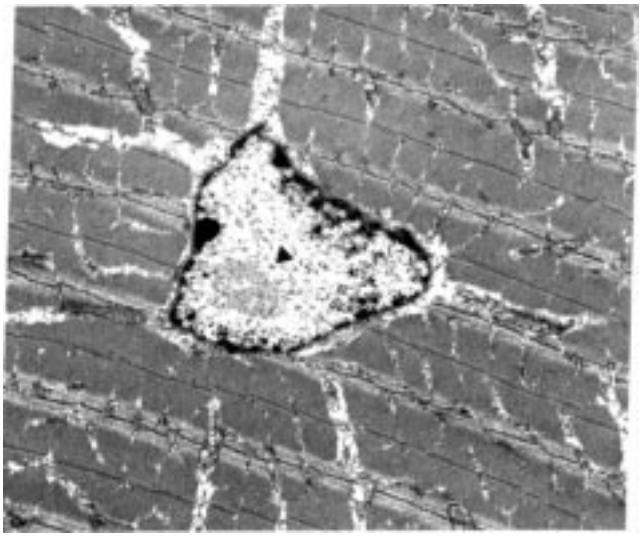

Figure 2. Ultrastructure of a nuclear inclusion (arrow) in the quadricep muscle of an R6/2 mouse at 12 weeks of age.

6. DISCUSSION

The molecular pathogenesis of polyQ disease is triggered by the gain of function that the polyQ expansion imparts to the protein in question. The gain of this new function must be correlated with the threshold at which the repeat expands into the pathogenic range, and any proposed molecular mechanism must account for the late onset of the disease. Neuronal inclusions have become the pathological signature of polyQ disease; the ability of expanded polyQ tracts to form amyloid-like aggregates provides an attractive molecular mechanism.

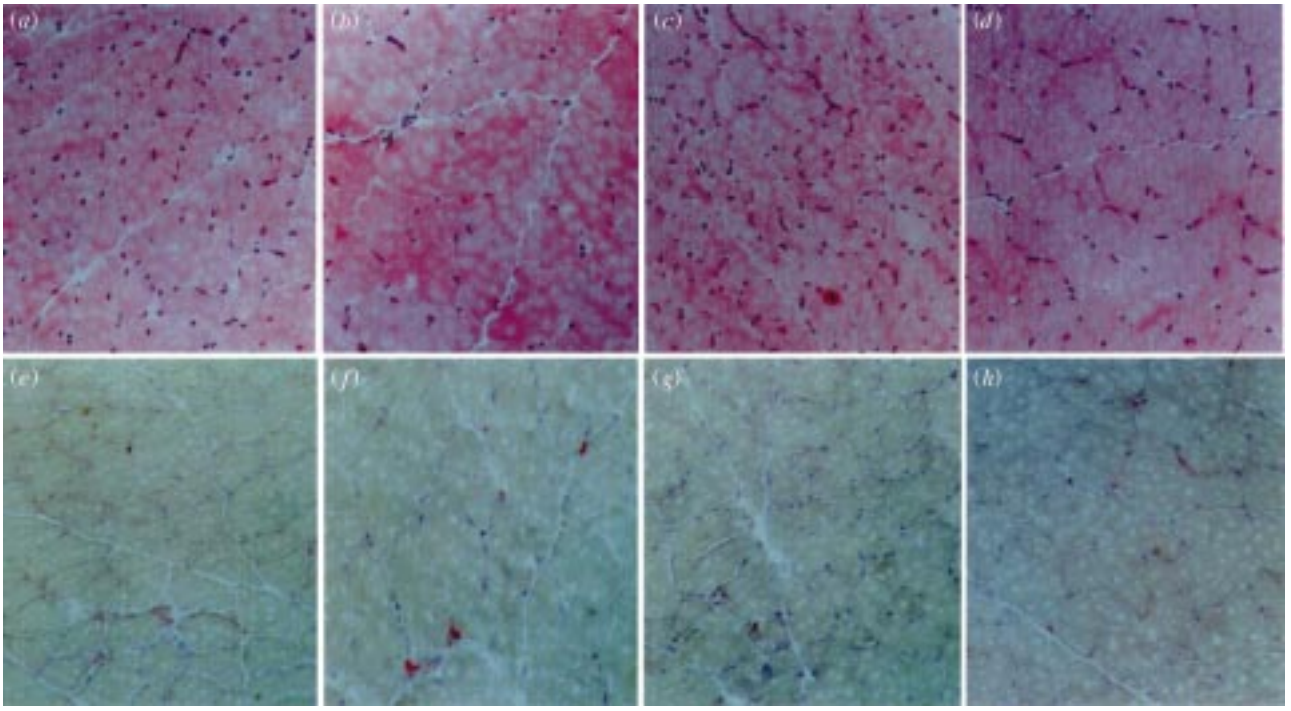

Figure 3. Analysis of the muscle atrophy occurring in the R6 transgenic lines. Transverse sections from quadricep muscle were stained with haematoxylin and eosin (H and E) (*a–d*) and for acid phosphatase activity (*e–h*). (*a*, *e*) Fifteen-month-old R6/1 transgenic animal; (*b*, *f*) 15-month-old non-transgenic control; (*c*, *g*) 14-week-old R6/2 transgenic animal; (*d*, *h*) 14-week-old non-transgenic control. The only difference between the transgenic animals and their normal littermate controls is a uniform shrinkage across all fibre types.

The formation of inclusions in neurons might occur as a result of the terminally differentiated nature of these cells, allowing the critical concentration for aggregation to be reached. A mitotically active cell might be expected to dilute the concentration of the precursor molecule at each cell division. The presence of nuclear inclusions in skeletal muscle in the R6 lines is consistent with this model. However, the occasional identification of inclusions in non-proliferating astrocytes (S. W. Davies, unpublished data) indicates that they can form in mitotically active cells with a slow turn over. Skeletal muscle inclusions were always nuclear, in contrast with the brain, in which neuritic inclusions were also identified at late stages in line R6/2 and in the more slowly progressing R6/1 line. The multinucleate structure of muscle fibres might predict that the transgene protein would never reach the concentration necessary for aggregation outside the nucleus.

An extensive histological analysis of skeletal muscle in the R6 lines was conducted to shed light on the basis of the extensive muscle atrophy that occurs in these mice. The decrease in muscle bulk was found to be caused by a uniform shrinkage of all muscle fibre types; no evidence for a myopathy or neuropathy could be found. In line R6/2, the appearance of inclusions at six weeks correlated with the onset of the muscle atrophy. However, by 14 weeks there was no evidence of any degeneration and the only difference between the transgene and control muscle at ultrastructure was the presence of nuclear inclusions. Degenerative changes were observed only after a protracted period, in this case in the form of non-specific ultrastructural changes in a 15-month-old R6/1 mouse. These findings are similar to those observed in brain. Despite the presence of inclusions in all neuronal classes by the end stage, very little selective neuronal degeneration can be observed. Therefore within the lifetime of the R6/2 mouse, the molecular pathogenesis leads to neurodegeneration in only a small subset of neurons.

It is not known whether inclusions form in HD skeletal muscle as there are no ultrastructural reports or examples of immunohistochemistry with anti-N-terminal huntingtin antibodies to HD post-mortem tissues outside the CNS. Several lines of evidence suggest that inclusions in post-mortem HD brains contain only an N-terminal huntingtin fragment (Becher *et al*. 1998; DiFiglia *et al*. 1997). The cleavage or proteolysis event that creates this fragment is unknown but it represents the first rate-limiting step in the pathogenic pathway. In generating the R6 lines, this first step has been bypassed. Therefore whether the pattern of inclusion formation observed in the R6 lines is recapitulated in the disease could be expected to depend on (i) any tissue variations in the distribution of factors involved in the generation of this fragment, (ii) comparative expression levels, and (iii) the length of the polyQ repeats. It will be necessary to look for inclusions in the skeletal muscle of juvenile patients to address this issue definitively. A recent study of the peripheral organs in SBMA identified non-CNS inclusions in scrotal skin, dermis, kidney, heart and testis but not in spleen, liver and muscle (Li *et al*. 1998). In this case, the cells containing inclusions were mitotic cells, capable of mitosis in adulthood. Inclusions were nuclear and contained the N-terminus of the androgen receptor (AR). Li *et al*. suggest that this pattern could not be explained by expression levels alone and could be influenced by the presence of a protease that cleaves the N-terminal fragment from the full-length protein, specific

proteins that interact with the mutant AR or the different AR functional activities in the different tissues.

A major effort is to be invested in the identification of small molecules that can prevent or slow down the kinetics of polyQ aggregate formation. These will be selected initially for their ability to prevent aggregation *in vitro* and then tested in the R6 lines as possible therapeutic agents. The identification of inclusions outside the CNS is important because it will allow the ability of drugs to prevent aggregation *in vivo* without the requirement in the first instance that the molecule in question can cross the blood–brain barrier.

We thank Stefan Buk and Hans Lehrach for discussions and Robin Poston for muscle fibre morphometry. This work was supported by grants from the Wellcome Trust, European Union (BMH4 CT96 0244), Hereditary Disease Foundation (in the form of an award to G.P.B. from Harry Lieberman), the Huntington's Disease Society of America and the Special Trustees of Guy's Hospital.

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